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Extracellular matrix metalloproteinase inhibitor (EMMPRIN), a plasma membrane glycoprotein identified originally in carcinoma cells, is responsible for inducing peritumoral fibroblasts to produce matrix metalloproteinases (MMPs), thereby enhancing cancer invasion and metastasis. Recently, it has become apparent that EMMPRIN is also involved in selective normal tissue functions. Using in situ hybridization, we have identified EMMPRIN in breast cancer cells. New monoclonal antibodies to EMMPRIN and MT1-MMP are being employed to further define their immunohistochemical localization in breast cancer. We have characterized the human EMMPRIN gene and have noted a high degree of conservation with the mouse gene. In the 5' flanking region, three SP1 and two AP2 sites were identified in the promoter region. The effect of transfecting human breast cancer cells with EMMPRIN cDNA was explored. Expression of EMMPRIN cDNA by MDA-MB-536 cancer cells resulted in a more malignant phenotype after intramammary injection of tumor cells in nude mice. The effect of EMMPRIN on endothelial secretion of MMPs was explored in vitro; EMMPRIN enhanced endothelial cell production of stromelysin-1, collagenase, and gelatinase A. These data support a role for EMMPRIN in cancer dissemination. The mechanism of action and regulation of EMMPRIN in malignant tissue remain to be examined.

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Stanley Zucker 12/24/97

PI - Signature Date

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INTRODUCTION

The focus of this grant is on the role of Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) in breast cancer invasion and metastasis. EMMPRIN is a glycoprotein identified on the plasma membrane of cancer cells which induces fibroblasts to produce Matrix Metalloproteinases (MMPs) (1).Originally known as Tumor Collagenase Stimulating Factor (TCSF) because it was described as a stimulator of interstitial collagenase (MMP-1) production by fibroblasts, TCSF was subsequently demonstrated to also induce cells to synthesize gelatinase A and stromelysin-1, hence the name change to EMMPRIN to reflect induction of synthesis of other MMPs.

EMMPRIN (TCSF), was initially purified from the plasma membranes of cancer cells by Biswas et al. and identified as a 58 kDa glycoprotein. EMMPRIN had no mitogenic activity and therefore differs from most well characterized cytokines, such as IL-1, TNF, and TGF-β. Monoclonal antibodies raised against EMMPRIN from human lung carcinoma cells were used to purify and characterize this membrane glycoprotein (2). The E11F4 monoclonal antibody also inhibited the biological activity of EMMPRIN, thereby proving that EMMPRIN was the effector molecule. Following development of specific monoclonal antibodies, EMMPRIN was identified by immunohistochemistry in the cell membranes of malignant epithelial cells from tumor specimens of human lung cancer and bladder cancer (3). Recent reports from other laboratories have confirmed the stimulatory effect of human breast and bladder cancer cells on production of MMPs by host fibroblasts (3, 4).

The central hypothesis of this grant is that breast cancer cells produce EMMPRIN which then induces peri-tumoral fibroblasts to produce the MMPs (collagenase, gelatinase A, and stromelysin-1) required for cancer dissemination. The biologic role of EMMPRIN in cancer was not initially appreciated because tumor cell lines grown in vitro generally produce high levels of MMPs, thereby suggesting that tumor cell production of MMPs was responsible for cancer invasion and metastasis. Fibroblast production of MMPs was relegated to a secondary role. In retrospect, this confusion seems to have been brought about because cancer cells that develop into immortalized cells in tissue culture generally produce high levels of MMPs. Cancer cells incapable of producing MMPs in vitro seem to be at a disadvantage in terms of developing into immortalized cell lines. Based on these observations, we postulate that high MMP production is a selection factor for enhanced cell growth in vitro. Hence, although the vast majority of cancer cells within a tumor do not produce MMPs in high levels, the few cells that produce MMPs have a growth advantage in tissue culture. These data may explain the lack of enthusiasm for a role for EMMPRIN in cancer dissemination until it was demonstrated that the MMP producing cells within a tumor are primarily host fibroblasts rather than cancer cells.

Considerable evidence has been presented in the past 3 years to support the Biswas concept that cancer cells signal fibroblast to produce MMPs (1). In situ hybridization studies of human breast cancer tissues have shown that the cDNA for matrix metalloproteinases (stromelysin-3, gelatinase A, interstitial collagenase, MT-MMP) is localized to fibroblasts surrounding the tumor rather than to the tumor cells themselves (5, 6) . Immunohistochemical studies using monoclonal and polyclonal antibodies, however, have identified gelatinase A in breast cancer cells suggesting that the tumor cells may bind this MMP to their cell surface (7-9). Interpretation of these data has led to the conclusion that normal host fibroblasts produce much of the MMPs that the cancer cells utilizes during invasion (10). The presence of gelatinase A in the cytosol of breast cancer cells, however, suggested to Hoythtya et al. (9) that membrane-bound gelatinase A may be internalized or that breast cancer cells, indeed, synthesize gelatinase A in vivo.

Using immunohistochemistry, we have demonstrated the selective localization of EMMPRIN on the surface of malignant cells in human breast cancer tissue (11), further suggesting that this factor may provide the missing link to explain the observation that peritumoral fibroblasts are the major producers of MMPs.

Based of the potential importance of EMMPRIN in regulation of MMP activity during tumor cell invasion, we have studied EMMPRIN at the molecular and physiologic level.

Following determination of the nucleotide sequence of the cDNA for human EMMPRIN (1), it was recognized that EMMPRIN is homologous to proteins of the Ig superfamily (basigin, neurothelin, OX-47, M-6) (12, 13) which have been identified in arthritis and embryonic epithelial/stromal interactions; the function of these proteins was not explored in these latter studies, but a function similar to EMMPRIN would be appropriate in these situations.

Summary of important publications since 1996 that are relevant to this grant

Scientific interest in EMMPRIN/basigin as a fascinating plasma membrane protein is of recent onset and emanates from laboratories studying diverse topics in cell biology. These studies are primarily descriptive and did not pursue the biologic function of EMMPRIN. Early studies used monoclonal antibodies to identify EMMPRIN/basigin as a plasma membrane antigen on endothelial cells localized at the blood brain barrier, hence the designation, "neurothelin". Subsequently. EMMPRIN/basigin was identified in cells in the blood, skin, and kidney (13). Although these investigators have acknowledged our demonstration of the biologic function of EMMPRIN in tissue remodeling, other investigators have not initiated studies of the MMP-inducing activity of EMMPRIN.

The studies described in this Section have led us to reassess some of the goals and priorities that were listed in our initial Army grant application. These modifications are described in the Body of the Annual Report.

1) Expression of EMMPRIN by non malignant cells

A novel monoclonal antibody that has been generated against a human leukemic T cell line (Molt 13) was found to recognize neurothelin/EMMPRIN (antigen recently termed CD 147). This antibody precipitated an antigen of 35-40 kDa in T cells, especially in thymocytes rather than peripheral blood T cells indicating that EMMPRIN is highly regulated during T-cell differentiation (14). The fact that Jurkat tumor cell EMMPRIN yielded a broad band in the range of 50-60 kDa suggests that extensive glycosylation may be more prominent in malignant cells as compared to normal cells. Based on our studies demonstrating non glycosylated EMMPRIN is ineffective in inducing MMP expression, we propose that EMMPRIN must be extensively glycosylated for functional activity as an inducer of MMPs. Therefore, it needs to be determined whether the 35-40 kDa EMMPRIN molecule has functional activity in T cells.

In another study a mouse monoclonal antibody directed against rabbit erythrocyte ghosts identified basigin/EMMPRIN (56-63% homology) in normal rabbit kidney tubule cells; the glycosylated EMMPRIN was expressed at the plasma membrane (15). Expression was regulated by interferon and serum. Erythrocyte and heart EMMPRIN appeared less glycosylated than kidney EMMPRIN. The presence of EMMPRIN in erythrocytes raises questions about modifications (i.e. glycosylation) of EMMPRIN required for function as an inducer of MMPs.

2) EMMPRIN knock-out mouse

In order to understand the in vivo function of EMMPRIN, Igakura et al. (16) produced knock-out mice lacking the EMMPRIN gene. The null mutant embryos mainly died around the time of implantation. A small number of surviving mice were sterile, indicating that the EMMPRIN gene is important in development and reproduction. These investigators then investigated the function of the EMMPRIN gene in the few surviving adult knock-out mice. The mitogenic response of lymphocytes from EMMPRIN knock-out mice was greater than normal in mixed lymphocyte culture. EMMPRIN knock-out mice had an abnormality in reception or response to odors.

3) Association of EMMPRIN with integrins at the cell surface

Recent studies examining the formation of complexes between integrins and other cell surface proteins using monoclonal antibody and cross-linking approaches have demonstrated that EMMPRIN/basigin is associated with $\alpha3\beta1$ and $\alpha6\beta1$, but not $\alpha2\beta1$ or $\alpha5\beta1$ integrins.

Immunofluorescent studies showed that EMMPRIN co-localizes with $\alpha3\beta1$ in cell-cell contacts and may interact with one another in a receptor-ligand fashion or in a lateral fashion (17). Using another approach involving the isolation of protruding plasma membrane structures from tumor cells, W.T. Chen (personal communication) has demonstrated that EMMPRIN is a major protein in invadopodia and is associated with integrins and MT-MMP in that location.

Other studies have shown that direct cell contact with cancer cells can induce fibroblasts to produce gelatinase B. MMP induction was blocked by anti-$\beta1$ integrin monoclonal antibodies suggesting that integrins and an organized actin cytoskeleton is required. An EMMPRIN- like mechanism was proposed, but no attempt to isolate the factor was described (18).

4) Expression of EMMPRIN in malignant skin lesions

Examining biopsy specimens from patients with skin cancers, it has been recently demonstrated that early invasion of malignant melanoma is associated with de novo expression of EMMPRIN and gelatinase B in malignant cells. It was suggested that the EMMPRIN stimulation of dermal fibroblasts to secrete various types of MMPs is among the factors involved in changes in matrix in the papillary dermis that are the earliest characteristic of tumor progression. EMMPRIN positive fusiform cells were also noted in cellular blue nevi. The authors proposed that EMMPRIN and gelatinase B may be partly responsible for the stromal changes observed in malignant melanoma; the absence of these proteins in the vertical growth phase and in metastatic lesions suggests that other factors are involved in tissue degradation during later stages of melanoma tumor progression (19).

5) Structure of the mouse EMMPRIN/basigin gene

The mouse EMMPRIN gene was reported to consist of 7 exons and 6 introns spanning 7.5 kb. The 5' upstream sequence of the mouse EMMPRIN gene contains no TATA box or CAAT box. Potential binding sites for NF1 and AP2 were observed (20). This study is quite relevant to our study of the human EMMPRIN gene (see below).

6) Cell surface localization of chicken EMMPRIN

Neurothelin, the homologue of human EMMPRIN on chicken endothelial cells at the blood-brain barrier, was studied in embryogenesis. The polarized cell surface distribution of neurothelin was shown to be influenced intracellularly by F-actin and extracellularly by cell-cell interactions. The possibility that glycosylation may provide a means to modulate protein function was raised. This statement is relevant to our work with human EMMPRIN (21).

7) Distribution of MMPs (focusing on membrane type 1- MMP) in breast cancer tissues

As described by our collaborators, Polette and Birembaut, numerous papers have recently confirmed the theory that we proposed in our original Army grant, indicating that in breast cancer, the peritumoral stromal cells (fibroblasts) produce and secrete gelatinase A, which could then be bound by tumor cells on their plasma membranes and used to degrade basement membrane and extracellular matrix (22). This data is consistent with our finding that EMMPRIN produced by breast cancer cells stimulates fibroblast production of MMPs. Polette and Birembaut further demonstrated that MT1-MMP, which is responsible for pericellular activation of progelatinase A, is also produced by stromal cells in parallel with gelatinase A. These authors (22) further demonstrated that EMMPRIN is primarily expressed in breast cancer cells located in the same area as the peritumoral fibroblasts that express gelatinase A. In relation to our Aim 1b (see below), this concept seems well proven and does not need further corroboration.

Polette et al. also demonstrated that conditioned media of invasive breast cancer cells (MDA-MB-231) induced MT1-MMP mRNA in human fibroblasts and a parallel activation of progelatinase A, whereas non invasive breast cancer cells (MCF-7) did not have any effect (23). This observation leads us to wonder whether the secreted form of EMMPRIN (lacking the transmembrane and cytoplasmic domains) retain biologic activity. Specifically, is secreted EMPIRE the inducer of MT1-MMP?

BODY OF ANNUAL REPORT

Experimental Results (the original timetable for these tasks is listed in parentheses)
Accomplishments achieved in years 1-2 (12/94 to 11/96) and year 3 (12/96 to 11/97) are identified separately.

Task (1). Identify the cellular localization of EMMPRIN (TCSF) and MMPs in human breast cancer tissue.

(1a) Obtain tissue samples from patients with various forms of breast cancer (24 months):

12/94 to 11/96- Breast tissue samples were obtained from 57 women with various forms of breast cancer and women with benign breast disease.

12/96 to 11/97- 30 additional breast tissue samples are now available for study which completes our original goal.

(1b) Immunolocalization of EMMPRIN and MMPs in human breast cancer tissue using specific antibodies to EMMPRIN to determine epithelial:mesenchymal contributions (36 months):

12/94 to 11/96- Using immunohistochemical techniques and our original E11F4 monoclonal antibody to EMMPRIN, we characterized the cellular localization of EMMPRIN as compared to the localization of gelatinase A in breast cancer. Tumor sections obtained from 28 women with breast cancer were examined. In all cases of invasive ductal cancer, antibodies to EMMPRIN reacted strongly with invasive cancer cells with intense staining of the plasma membrane and less intense staining of cytoplasm. In comparison with cancer cells, normal ducts within the tumor specimen demonstrated similar staining with anti-EMMPRIN antibody. EMMPRIN immunostaining was intense in both early and advanced stages of invasive breast cancer, as well as in situ breast carcinomas. Moderate EMMPRIN staining of breast ducts and acini was noted in breast tissue obtained from biopsies of patients with benign breast disease and normal breast tissue (obtained from mammary reduction surgery). Colored photos were displayed in the 1996 report.

Limitations in quantifying EMMPRIN antigen concentrations in human tissue using the E11F4 monoclonal antibody could not be ruled out. These data suggested that EMMPRIN may have a function in embryonic development or maintenance of normal breast tissue, as well as the malignant process. We propose that in physiologic processes, the presence of an intact basement membrane separating the normal/benign epithelium from underlying stromal fibroblasts limits access of epithelial cell EMMPRIN for induction of MMP production by stromal fibroblasts through a cell-cell contact related mechanism. In contrast, in carcinomas the epithelial basement membrane is fragmented, thereby permitting epithelial cancer cells to migrate into the stroma, make direct cell contact with fibroblasts, and stimulate fibroblast synthesis of MMPs. The enhanced production of MMPs by peritumoral fibroblasts then leads to degradation of the stroma (including the basement membrane), thereby enhancing the invasive/metastatic process of cancer cells.

12/96 to 11/97- The discrepancy between our results on immunohistochemistry and in situ hybridization (see below) have led us to plan to repeat the immunohistochemical studies using a different monoclonal antibody to EMMPRIN. These studies will be done in 1998 using one of the new monoclonal antibodies described below. If studies using the new antibody to EMMPRIN confirm the results with E11F4, we will need to design other experiments to clarify the dilemma. The possibility that the E11F4 monoclonal antibody recognizes another member of the IG superfamily besides EMMPRIN may need to be considered and tested for appropriately. One would hope that the new monoclonal antibodies to EMMPRIN that we have recently isolated may not be subject to the same cross reactivity; therefore, if the data with E11F4 is confirmed, this will strengthen the conclusion that EMMPRIN protein is present in high concentration in normal breast ducts.

(1.b.1) Production of new monoclonal antibodies to EMMPRIN

12/94 to 11/96 - Production of monoclonal and polyclonal antibodies to recombinant EMMPRIN for use in ELISAs. Eight BALB/c mice were immunized by the intraperitoneal injection of recombinant EMMPRIN (purified from CHO cell homogenates transfected with EMMPRIN cDNA). In spite of the development of high serum titers of antibodies against the immunogen in mice, antibodies adequate for ELISA were not produced. The problem with the initial 20 mouse myeloma clones developed was that all of the antibodies were of the IgM type and reacted nonspecifically with other proteins as demonstrated by Western blotting.

12/96 to 11/97 - To circumvent these problems, we used EMMPRIN purified from human lung cancer cells (LX-1) rather than recombinant EMMPRIN as the immunogen. EMMPRIN antigen injections (100 ug) in mice were performed on a 2 week schedule and mice were sacrificed after 8 weeks. This work was done in collaboration with Chemicon Corp. (Drs. Dale Dembro and Alex Strongin, San Diego, CA). Antibodies to EMMPRIN in mouse serum were present at a titer of 1:10,000 using native antigen rather than recombinant antigen. Spleen myeloma cells fusions growing in splenocyte conditioned media resulted in the production of 63 positive IgG producing wells. The 15 clones with the highest antibody titers as detected by EIA (20 ng EMMPRIN per well) were subcloned with 8 active clones identified after two subcloning procedures. All 8 clones produced distinct, broad bands (a result of extensive glycosylation) at ~53 kDa on Western blotting with EMMPRIN (Figure 1); weaker bands presumably representing degradation products of EMMPRIN were also noted (additional studies required). Non-specific cross reactivity with other proteins was not a problem.

(1.b.2) <u>New Aim: Production of monoclonal antibodies to Membrane Type (MT1)-MMP</u>
Since the description of MT1-MMP in 1994 (24, 25) , it has become apparent that this membrane bound MMP is important in cancer because it functions to activate progelatinase A in the pericellular environment. We propose that following the induction of progelatinase A synthesis by EMMPRIN, MT1-MMP is required for activation of progelatinase A. As with most MMPs, MT1-MMP has been described by in situ hybridization as being expressed in tumoral fibroblasts rather than epithelial cancer cells (26). Since our previous study demonstrated that EMMPRIN treated fibroblast secreted activated as well as latent gelatinase A (27), this data suggested that EMMPRIN may also be responsible for inducing the synthesis of MT1-MMP. Based on these observations we have developed high titer monoclonal antibodies that react with MT1-MMP by injecting recombinant human MT1-MMP (fusion protein with GST) lacking the transmembrane domain (protein produced in bacteria) into Balb/c mice. Following fusion of mouse spleen cells with immortalized myeloma cells, antibody producing clones were selected. These antibodies will be employed in the immunohistochemistry studies of breast cancer described below. Two of the 30 monoclonal antibodies that we developed are excellent capture antibodies for biotinylated MT1-MMP. Six of these monoclonal antibodies demonstrate high titer efficiency as detecting antibodies for immobilized MT1-MMP. We are currently in the process of pairing the optimal capture and detecting antibodies for use in a MT1-MMP ELISA which will be performed on breast cancer tissue homogenates.

(1c) <u>Develop an ELISA for EMMPRIN (TCSF) for use in quantifying antigen in breast tissue</u> (6 months):
12/96 to 11/97 - The development of an ELISA for EMMPRIN has been delayed until now because of the absence of antibodies of sufficiently high titer and affinity for EMMPRIN. Purified EMMPRIN (100 ug) has recently been biotinylated and is being used to determine which of the monoclonal antibodies described in Section 1.b. 1 functions best as a capture antibody in an ELISA. Each of the monoclonal antibodies has been immobilized in 96 well plates and after washing and blocking steps, biotinylated EMMPRIN was added for 1 hour. Streptavidin- alkaline phosphatase and substrate were used for color development. The results of these experiments is currently being analyzed. The monoclonal antibody with the highest anti-EMMPRIN titer against immobilized EMMPRIN will be biotinylated for use as the detecting antibody in the ELISA (studies ongoing). Checkerboard analysis of optimal antibody

concentrations will be performed. Breast cancer tissue stored in liquid nitrogen will then be tested by ELISA for EMMPRIN content as initially planned.

(1d) Quantify the EMMPRIN, gelatinase A, gelatinase B, and stromelysin-1 content of fresh tissue samples obtained from patients with breast cancer (36 months):

Studies in progress- In addition to testing for gelatinase A and gelatinase B as initially proposed, tissue samples will also be tested for MT1-MMP (see above for antibody development). Tissue samples will be thawed in batches and tested for each of the MMPs as initially planned. Based on several recent studies demonstrating that stromelysin-1 is not expressed in high concentrations in breast cancer (26), this ELISA will not be performed.

An anticipated limitation to these studies will be the sensitivity of our ELISAs for detection of small amounts of MMPs in stored tissue samples.

(1e) Identify mRNA for EMMPRIN (TCSF) in breast cancer tissue using in situ hybridization (48 months):

12/94 to 11/96- This task was completed ahead of schedule (publication enclosed). To characterize and distinguish the cells producing EMMPRIN and gelatinase A in breast cancer, we employed in situ hybridization using radiolabeled RNA probes for EMMPRIN and gelatinase A (28). Surgical specimens were obtained from 22 women with breast cancer and from 7 women with benign breast disease (fibrocystic disease and fibroadenoma). The result of these studies was that EMMPRIN mRNA was detected by in situ hybridization in all carcinomas in both non invasive and invasive cancer cells and in pre malignant areas such as atypical hyperplasia of the breast. EMMPRIN mRNA and gelatinase A mRNA were both visualized in the same areas in serial sections in breast cancer, but were expressed by different cells with tumor cells expressing EMMPRIN mRNA and fibroblasts expressing gelatinase A mRNA. There was no correlation between EMMPRIN mRNA and the tumor size, grade of the tumors, the number of lymph node metastases, and the hormonal receptor status of the tumors. Normal mammary glands adjacent to cancer areas showed no EMMPRIN hybridization grains. By Northern blot analysis of tissue extracts, higher expression of EMMPRIN mRNAs in breast cancers was noted as compared to benign and normal breast tissue; however, the normal tissue expression was not negative as noted by in situ hybridization (see enclosed manuscript for figures). The discrepancy noted between normal breast ducts staining positively for EMMPRIN by immunohistochemistry but negatively by in situ hybridization suggested differences in sensitivity of these techniques for EMMPRIN detection or differences in the rates of EMMPRIN turnover in normal versus malignant tissue (29).

Task (2). Identify important structural:functional relationships in the EMMPRIN (TCSF) molecule.

(2a) Determine whether post-translational processing is required for biological activity (12 months): **This task has been completed.**

12/96 to 11/97 (publication enclosed)- To circumvent problems related to the requirement for glycosylation of EMMPRIN for function of the protein, we stably transfected mammalian cells (CHO) with EMMPRIN cDNA. Posttranslational processing resulted in the production of EMMPRIN of molecular weight identical to native EMMPRIN from tumor cells i.e. ~58 kDa. We immunopurified the recombinant EMMPRIN after extraction from CHO cell membranes using monoclonal antibodies raised against native tumor cell EMMPRIN. When added to human fibroblasts in culture, the purified recombinant EMMPRIN was found to be active in stimulating production (2-5 fold) of fibroblast interstitial collagenase, gelatinase A, and stromelysin-1, but not TIMP-1 (30). Since non-glycosylated and partially glycosylated recombinant EMMPRIN were unable to stimulate MMP production, we conclude that post-translational processing is required for EMMPRIN activity.

New Task- Since we know that post-translational processing is essential for EMMPRIN activity, we plan to prepare mutant EMMPRIN protein with altered glycosyation sites to learn more about the specific glycosylation requirements for EMMPRIN bioactivity in inducing

MMP synthesis. This work will begin in 1998.

(2b) Alter EMMPRIN (TCSF) by deletional mutation and site directed mutagenesis of cDNA and then analyze mutant proteins to determine the minimum amino acid sequences necessary for functional activity (36 months):

(2.b.1) We reconfirmed the identity of the EMMPRIN cDNA by showing that recombinant protein is recognized by the activity blocking monoclonal antibody (E11F4). Two immunoreactive bands were obtained corresponding to the forms previously noted (1). The bacterial recombinant proteins was also used to determine the approximate location of the epitope for E11F4, taking advantage of the lack of posttranslational modification that would interfere with such studies on the native, immunoaffinity-purified protein. Modified EMMPRIN expression plasmids were made containing deletion in four locations. XL-1 blue cells were transformed with the deletions expression pBluescript plasmids, and the expressed proteins were analyzed by SDS-PAGE and Western blotting with monoclonal antibody E11F4. All of the plasmids produced protein that is immunoreactive, except for the plasmid lacking immunoglobulin domain I (see enclosed manuscript). These results demonstrate that our cDNA encodes the protein which is reactive with our activity-blocking monoclonal antibody and that the antibody epitope exists in the extracellular immunoglobulin domain I. This, in turn, implies that the functional site of the metalloproteinase stimulatory activity of EMMPRIN is likely to be localized to sequences contained in the immunoglobulin domain I region.

The next step is to express the polypeptides that lack either domain I or domain II of the extracellular region of EMMPRIN in plasmid pCM for transfection in COS 7 cells. As we previously demonstrated, EMMPRIN produced in bacteria will not be useful for examining bioactivity since the non glycosylated protein does not induce MMP production. The biologic function of these COS 7 proteins in inducing MMP synthesis in fibroblasts will be assessed. More restrictive mutations by the same approach will be produced to narrow down the peptide regions responsible for bioactivity as originally described.

(2.b.2) Characterization of the human EMMPRIN gene (previously identified as new task 5 in 1996 Annual Report)

As a result of our sequencing of keratinocyte cDNA for EMMPRIN and its strong homology with cancer cell EMMPRIN, and the identification of EMMPRIN in normal rabbit kidney cells, T lymphocytes, and erythrocytes, we considered the strong possibility that the difference between the high level of synthesis of EMMPRIN in cancer cells and the apparent low level synthesis in non malignant cells may be due to differences in the promoter region for EMMPRIN, specifically in the mixed response element category. This led us to undertake the sequencing of the entire human EMMPRIN gene as a priority goal.

cDNA hybridization have been used to determine the chromosomal location of both mouse and human EMMPRIN/basigin genes, mapping the mouse to chromosome 10 and human to 19p13.3.

12/96 to 11/97- We herein present the first isolation and characterization of the human EMMPRIN gene (Figure 2). The probes used for S1 nuclease analysis are listed in Figure 3A. the S1 nuclease analysis to determine the transcription start site is listed in Figure 3B. While a high degree of conservation is seen between the human and mouse gene structures, a notable difference is that the human protein is encoded by 8 exons (Figure 4), while that of the mouse is encoded by 7. The sum of the exon and intron sizes is 10.8 kb in the human, and 7.5 kb in the mouse. Using EMMPRIN cDNA, we have isolated a cosmid clone that contained the EMMPRIN gene. S1 analysis with a fragment of the gene was performed to determine the transcription start site. PCR and sequence analysis has defined the exon/intron organization of the gene and shows that it is highly conserved with the mouse EMMPRIN/basigin gene. Figure 5 is a schematic diagram of the mRNA, showing exon orders and domain structures. Starting from left to right is the 5' UTR and the signal peptide encoded by Exon 1. The two half circles indicate the Ig domains of the extracellular portion of the molecule. About 950 bases of the 5'

flanking region were examined for transcription factor consensus binding sites, locating three SP1 sites and two AP2 sites. AP2 and SP1 sites overlap in the EMMPRIN gene. Of interest, in SV-40 the AP2 and SP1 sites also overlap and turn on the early genes. The early genes make T antigen which binds to the AP2 site and appears to turn off the early genes and remove AP2 from continued interaction with SP1.

The transcriptional start site for EMMPRIN was found to be located in a CpG island. In housekeeping genes, these multiple CG dinucleotides are unmethylated and are thought to play a role in their constitutive expression. No consensus TATA or CAAT boxes were observed in either the human or mouse gene.

Elements in the proximal promoter region were conserved in human and mouse genes. Figure 6 is a schematic of the 5' flanking regions of the human and mouse EMMPRIN genes. The human and mouse genes have the unusual property that each Ig domain is not encoded by one exon, but by two. Also unusual in the EMMPRIN gene as compared to other Ig family members is the fact that the downstream exon of the second Ig domain is a junctional exon encoding the transmembrane domain and part of the cytoplasmic domain as well. Most members of the Ig superfamily encode the Ig domain in a single, unshared exon.

These data lead us to postulate that when cells become transformed they might produce more SP1 and AP2 and those are the factors which bind to the EMMPRIN promoter, thereby enhancing the transcription and subsequent translation of EMMPRIN.

(2c) <u>Design peptide antagonists and produce anti-functional monoclonal antibodies to further characterize the structure:functional relationship of the EMMPRIN molecule</u> (48 months):

This task will be initiated in 1998 as planned.

Task 3. <u>Explore the role of EMMPRIN (TCSF) in cancer dissemination using experimental models</u>.

(3a) <u>Compare the effect of transfecting breast cancer cells with cDNA for native versus mutant EMMPRIN in regards to altering cancer invasion and metastasis in an experimental model</u> (48 months):

12/94 to 11/96- The open reading frame for EMMPRIN cDNA was transfected into human breast cancer cell lines (MDA-MB-436 and MCF-7). Transfected cell lines were then injected into the mammary fat pad of 6-8 week old nude female mice at a cell concentration of 1 X 10^6 per animal. Palpable tumors (1-4 cm in diameter) were detected after 3 months in 2/4 mice injected with EMMPRIN cDNA- transfected MDA-MB-436 cells and in 3/4 mice with mock transfected MDA-MB-436 cells. Metastases were noted in the local lymph nodes of 1/4 mock transfected cells, but not in EMMPRIN-transfected cells. Tumor size between EMMPRIN and mock transfected tumor injected animals were comparable. Histology of the breast cancers was as anticipated indicating that the morphologic phenotype had not changed. This experiment was subsequently refined in 1997 as described below.

12/96 to 11/97- To ascertain that transfected tumor cells are expressing EMMPRIN protein in high concentration, we took advantage of the recent observation that the green fluorescent protein (GFP) of the jelly fish Aequoria victora retains its fluorescent properties when recombinantly expressed in eukaryotic cells (31). This 29 kDa protein can then be used as a powerful marker for gene expression in vivo (32). To this end, we expressed EMMPRIN cDNA along with the GFP reporter cDNA as a fusion gene controlled by a CMV promoter in pcDNA3 expression vector. The GFP-EMMPRIN fusion protein was expressed in the plasma membrane of transfected COS-1 cells as documented using fluorescent microscopy, thus indicating that GFP is transported to the plasma membrane along with the EMMPRIN protein. A second plasmid was produced in which GFP and EMMPRIN are controlled individually by separate CMV promoters. A control plasmid containing GFP alone was also produced. We documented EMMPRIN expression in transfected cells by fluorescent microscopy and immunoblotting of cell lysates.

MDA-MB-436 human breast cancer cells were transfected with GFP cDNA and EMMPRIN/GFP fusion cDNA. Stable cell lines were selected using G-418. After 5 weeks, stably transfected cells were examined by Northern blotting. The expression of EMMPRIN mRNA in EMMPRIN transfected MDA-MB 436 cells was approximately 4 fold higher than in vector transfected cells (Figure 7). 1 x 10^7 tumor cells derived from these transfected cell lines were then injected into mammary tissue of 4 month old nude female mice. Mice were examined weekly thereafter for tumor size measurements.

Results: EMMPRIN/GFP transfected mice (7/10) developed breast tumors at the site of mammary injections that grew to 1.4-2.3 cm diameter within 7-12 weeks (Figure 8); intra-abdominal metastases were extensive in most mice (retroperitoneal, perigastric, sub diaphragm, kidney, etc.). These tumors were highly vascularized with numerous grossly visible vessels entering the tumor mass from normal tissue. GFP expressing tumors were green in color when examined by fluorescent light. Lung metastases were not observed in any of the groups. By comparison, the GFP (only) transfected tumor cells produced small, slow growing, poorly vascularized tumors (3/4 mice) that reached a diameter of ~2-3 mm in 12 weeks (not visible until autopsy). A complicated aspect of this experiment design was that mice injected with non-transfected MDA-MB 436 cells developed local breast tumors at approximately the same rate (3/4 mice) as mice injected with EMMPRIN/GFP fusion cells; the non-transfected cancer cells, however did not display extensive intra-abdominal metastases and were not highly vascularized. Further study of transfected cells (either GFP or EMMPRIN/GFP fusion cDNA) indicated that both sets of transfected cells propagated in vitro at a somewhat slower rate than non-transfected MDA cells and did not form foci to the same extent as non-transfected MDA-MB-436 breast cancer cells. This experiment suggests that: 1) transfection of the pcDNA expression vector into MDA-MB-436 cells resulted in partial loss of tumorigenic properties of these breast cancer cells; 2) transfection with EMMPRIN restored the malignant phenotype to MDA-MB-436 cells and resulted in intra-abdominal metastases and extensive tumor neovascularization; and 3) in the background of this less malignant transfected phenotype, expression of EMMPRIN cDNA resulted in more invasive and locally metastatic cancer cells. The fact that only 70-75% of the injected mice developed tumors suggests that technical problems may have limited the percentage of successful tumor engraftment.

These experiment are in the process of being repeated in order to determine the reproducibility of these observations. Groups of 12 nude female mice (5 weeks of age) have been injected with 10^7 transfected MDA-MB-436 cells into mammary tissue as described above. Tumor formation and metastases are in the process of being recorded.

Plan for 1998- A second breast cancer cell line will be examined to determine whether the EMMPRIN transfection effect is reproducible in other cell lines. Transfection with EMMPRIN cDNA into MDA-MB-435 cells and HS-578T (widely metastatic cell lines supplied from Georgetown University) has been performed as described above for MDA-MB-436. To simplify the interpretation of results, the GFP cDNA will not be included in this experiment. Tumor growth and metastasis of these cells as compared to non-transfected and vector-transfected cells will be examined.

(3b) Analyze human peritumoral fibroblast response to EMMPRIN (TCSF) in vitro (36 months):

The experiments described in the original grant will be initiated in 1998.

12/96 to 11/97 - Based on additional information available about EMMPRIN since 1994, it would appear that identifying the receptor for EMMPRIN on fibroblast target cells is a priority in understanding the mechanism of action of EMMPRIN and in determining whether tumor derived fibroblasts are more responsive to EMMPRIN's stimulatory effect than non activated fibroblasts. Based on these considerations, we have performed receptor binding studies with EMMPRIN.

Human HFL (lung fibroblasts) were metabolically labeled with ^{35}S methionine (200

uCi/ml) for 5 hours. Cells were washed thoroughly to remove unincorporated isotope. Purified human EMMPRIN from LX-1 cells (100 nM) was added to cells for 1.5 hours. The cross-linking reagent, 0.5 mM DTSSP (Pierce Co.) was added for 30 minutes at 23° C. The cells were then washed thoroughly and cells were lysed in PBS buffer with 0.1% triton X-100 detergent. The cell lysate was incubated with anti-EMMPRIN antibody (E11F4) overnight and then added to protein A agarose beads for 1 hour. The beads were then washed six times with PBS and boiled with 1x sample buffer for 5 minutes. The samples were loaded on a 10% SDS-PAGE and exposed to XRay film. The results demonstrate the binding of EMMPRIN to a 58 kDa and 42 kDa proteins on fibroblasts (Figure 9).

We next examined the binding of EMMPRIN to fibroblast membranes. Membrane extracts from human fibroblasts were loaded on an EMMPRIN (0.5 mg) affinity column (Carbolink or Aminolink column, Pierce) for 30 min. After washing the column with PBS, the protein was eluted from the affinity column with elution buffer and the samples were analyzed by SDS-PAGE followed by silver staining . As shown in Figure 10, a single protein of 58 kDa was identified. These results support the concept that fibroblasts contain a binding protein (receptor) for EMMPRIN that has a mass of ~58 kDa. Based on this data, we will now be able to compare quiescent fibroblasts versus tumor derived fibroblasts for their ability to bind to EMMPRIN. These results should coincide with the comparison of the biologic inducing effect on MMP synthesis of EMMPRIN on tumor fibroblasts as planned in the initial grant.

We are also in the process of isolating a sufficient amount of the affinity bound protein to determine the N-terminal amino acid sequence of this proposed EMMPRIN receptor on fibroblasts. These experiments will be done in 1998.

(3.c) New Task: Effect of EMMPRIN on production of MMPs by endothelial cells:

In the four years since this grant was written, it has become apparent that tumor angiogenesis plays an important role in the progression of cancer. An obvious question to be addressed is whether the tumor cells produce additional factors besides VEGF that would enhance tumor angiogenesis. Based on our demonstration that EMMPRIN produced by cancer cells stimulates fibroblasts to produce MMPs, we reckoned that EMMPRIN may have a similar effect on endothelial cells.

12/96 to 11/97- To address this question we have incubated EMMPRIN with endothelial cells to determine the effect on MMP production. EMMPRIN purified from stably transfected CHO cells also stimulates production of stromelysin-1, interstitial collagenase, and gelatinase A (demonstrated by ELISA) by human umbilical vein endothelial cells cultivated in vitro. EMMPRIN did not significantly enhance TIMP-1 secretion (Figure 11). By comparison, VEGF had a greater stimulatory effect than EMMPRIN on HUVEC synthesis of interstitial collagenase and TIMP-1, but not gelatinase A or stromelysin-1 (33). We concluded that EMMPRIN plays a role in the early phase of tumor angiogenesis by inducing the degradation of endothelial basement membrane. We propose that in vivo, direct contact between circulating tumor cells and endothelial cells lining blood vessels at organs distant from the primary tumor may facilitate tumor cell penetration of the subendothelial basement membrane during metastasis. In this scenario, tumor cell EMMPRIN induces endothelial cells to secrete MMPs that subsequently facilitate subendothelial basement membrane degradation; tumor cells then are able to migrate through the rents in the blood vessel wall (34). Additional studies are underway to examine whether the effects of EMMPRIN and VEGF are additive which would suggest that their mechanisms of action are independent of one another.

Our hypothesis that EMMPRIN is a positive factor in tumor angiogenesis is supported by our recent studies demonstrating more vascularized tumors produced by EMMPRIN-transfected breast cancer cells (see Task 3).

Task 4 (completed) added to original grant application (see 1996 annual report)
Human Keratinocyte EMMPRIN.

12/95 to 11/96- Although initial studies suggested that EMMPRIN is not present in significant amounts on many types of normal adult human cells (2, 3), it subsequently became

apparent that EMMPRIN is identical to human basigin and M6 antigen, (12, 13) and is expressed in some physiologically active epithelia during embryonic development, as well as tumor cells. Since keratinocytes have previously been shown to stimulate MMP production by fibroblasts (35) and since epithelial-dermal interactions are important in preserving and repairing skin structures, we sought evidence for the presence of EMMPRIN in keratinocytes. We found that human keratinocytes express EMMPRIN at their cell surface in vivo and in vitro and synthesize EMMPRIN in culture, albeit at a lower level than tumor cells. On characterization of EMMPRIN cDNA obtained from a keratinocyte cDNA library, we found that the deduced amino acid sequence was identical to that of tumor cell EMMPRIN (36). These cDNAs share a common region of 1459 nucleotide residues that differ in only 7 of these residues, only two of which are in the open reading frame and which result in no differences in the amino acid sequence of EMMPRIN. The significance, if any, of the polymorphism in the cDNA sequence of the two clones is unclear. We concluded that human keratinocytes produce EMMPRIN. A role for EMMPRIN at epithelial dermal junctions in tissue repair during wound healing seems highly plausible. Taken together, these data suggest that the function of EMMPRIN is under strict regulation in normal tissues, but this control mechanism may go awry in cancer.

CONCLUSIONS

EMMPRIN (TCSF) is a plasma membrane glycoprotein that is present on the surface of breast cancer cells, and is responsible, in part, for the elevated levels of MMPs in peritumoral fibroblasts and endothelial cells. EMMPRIN requires post-translational processing (glycosylation) for its ability to stimulate production of MMPs by target fibroblasts. Transfection of EMMPRIN cDNA into CHO cells resulted in the production of a glycosylated functional protein of similar molecular weight to native EMMPRIN (58 kDa) that was localized to the plasma membrane. EMMPRIN mRNA is expressed in benign and malignant human mammary ducts and acini to a much greater degree than in normal breast ducts. Immunohistochemical approaches, however, have indicated that EMMPRIN is also present in normal breast ducts and some other epithelial structures (i.e. T-cells, kidney tubules, endothelial cells, keratinocytes). The role of EMMPRIN in normal cells remains to be determined. The discrepancy between in situ hybridization and immunohistochemistry may be clarified by development of an immunoassay for tissue EMMPRIN. We have developed 8 IgG monoclonal antibodies to human EMMPRIN which will be employed in immunohistochemistry and immunoassay. We have characterized the human EMMPRIN gene. The sum of the exon and intron sizes is 10.8 kb. Transcriptional factor consensus binding sequences have been identified. We have transfected EMMPRIN cDNA into human MDA-MB-436 breast cancer cells and have demonstrated that these cells are more tumorigenic and invasive than plasmid transfected cancer cells. Dual transfection with EMMPRIN/GFP cDNA is described; cancer cells exhibit green fluorescence which makes it easier to detect metastatic foci. Studies were performed to identify a binding site for EMMPRIN on fibroblasts; a putative binding protein of 58 kDa was identified using 2 different experimental approaches. An inducing effect of EMMPRIN on endothelial cell synthesis of MMPs was identified. Based on studies to date, we propose that inhibition of EMMPRIN function may provide a potential mechanism to alter the invasive process in breast cancer.

FIGURES

Figure 1: <u>Western blot demonstrating the detection of EMMPRIN by mouse monoclonal antibodies</u>. Lanes were loaded with 25 ng human EMMPRIN purified from the membranes of LX-1 lung cancer cells, run on SDS-PAGE and transferred to nitrocellulose paper. After blocking and washing steps, mouse monoclonal antibodies from hybridoma supernatants were incubated with nitrocellulose strips and antibody was identified using the chemiluminescent method (Amersham).

<u>Anti Emmprin Western Blot Results</u>

16 December 1997

Anti mouse IgG(γ) alkaline phospnatease KPL TJo36 (1/1500)

Sample designation:

10	11	12	13	14	15	16	17	18
PBS	Nor Mu 1/100	MuxEmmprin Serum 1/1000	7F11.2 Super	6C5.2 Super	8D8.2 Super	3F3.2 Super	1G6.2 Super	4B9.2 Super

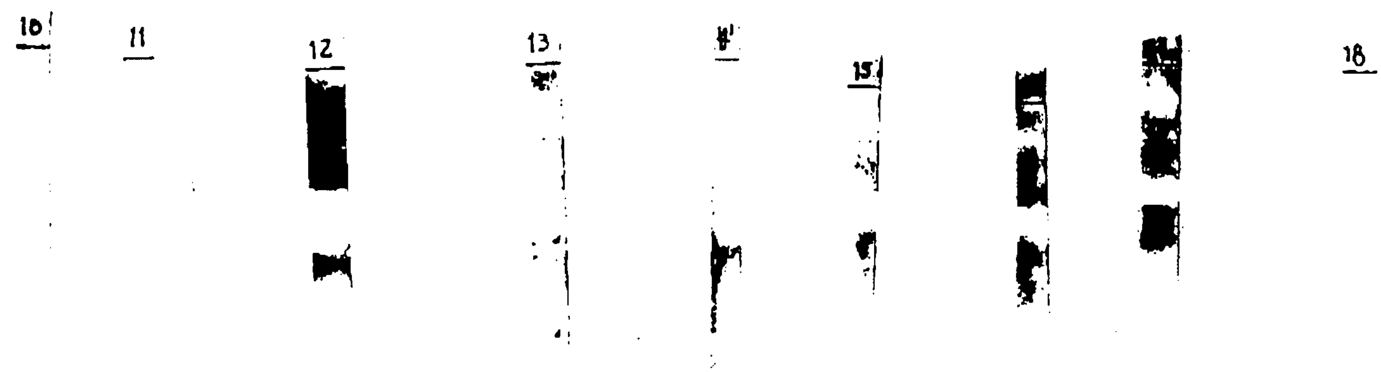

Figure 2: Sequence of the EMMPRIN gene and 5' flanking region. The transcriptional start site is indicated by a +1. The sequence includes 940 bp 5' of the transcriptional start site, all the exonic sequences, and the borders of introns. The 5' primer used in S1 nuclease analysis are indicated by double underlines. Primers used to determine the exon/intron organization are single underlines. Exonic sequences are in upper case letters; intronic are in lower case.

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-940  ggtcccgcaa agagaaacct gcacttagaa aatttctcag cgagaccact ttacttgtac
-880  ggaaggggc tgcctgcgtg ggatgcaatc tccagagcac acgggacaaa ggaagggagc
-820  ttctgttcct ctgtcctttc cctgttggct ggggttggac gccacaatct aagctgatcc
-760  cggttggcta aaacttaaaa ctttttaaaa tagggtaaac cggcacctcg gaggaaagaa
-700  gaagggggta aggttgattg acaactttag acctggaagt tgagtttctg aagaggaact
-640  tacttgtccc aaccaaatgg tgcacaggac gtttatacta agaaattatt cattttctga
-580  ggatccagat tcaagtgccc tttaagtatt tttatttggg cggaggaggt cacttcctcc
-520  cacctaattt attaataccc atatatatat atatatgtac attttaaag gcagagatgt
-460  gcgtctcgca tgtgccgcgt ctggtctcga gctcccgggc tcaagcgatc ttcccgcctc
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-220  ccccactccc gttcctagc aacgccgggt cacgtgcgcc gccgcccgga ttccgtagcg
-160  tgagcctgcc ggagccggcg cgtacatgcg agcgtgtgcg cgcgtgcgca ggcgggcgac
-100  cggcgtcccc gcgctcgccc cgcccccgag atgacgccgt gcgtgcgcgc gcccggtccg
                                                  +1
-40   cgcctccgcc gctttttata gcgccgcggg cggcggcggc agcggttgga ggttgtagga
21    ccggcgagga ataggaatcATG GCG GCT GCG CTG TTC GTG CTG CTG GGA TTC GCG CTG CTG
                          M   A   A1A   L   F   V   L   L   G   F   A   L   L    14
82    GGC ACC CAC GGA GCC TCC GGG GCT GCC Ggtgaggagcg—6.5 Kb—cttcagccagGC ACA GTC
       G   T   H   G   A   S   G   A   A       Intron 1              G   T   V    26
TTC ACT ACC GTA GAA GAC CTT GGC TCC AAG ATA CTC CTC ACC TGC TCC TTG AAT GAC AGC
 F   T   T   V   E  2D   L   G   S   K   I   L   L   T   C   S   L   N   D   S   46
GCC ACA GAG GTC ACA GGG CAC CGC TGG CTG AAG GGG GGC GTG GTG CTG AAG GAG GAC GCG
 A   T   E   V   T   G   H   R   W   L   K   G   G   V   V   L   K   E   D   A   66
CTG CCC GGC CAG AAA ACG GAG TTC AA gtgagtgcct—0.7 Kb—tgttgcagGGTG GAC TCC
 L   P   G   Q   K   T   E   F   K         Intron 2               V   D   S      78
GAC GAC CAG TGG GGA GAG TAC TCC TGC GTC TTC CTC CCC GAG CCC ATG GGC ACG GCC CAG
 D   D   Q   W   G   E   Y  3S   C   V   E   L   P   E   P   M   G   T   A   N   98
CTC AAC ATC CACGgtgagtctgc—0.3 Kb—ccctcctgtcagGG CCT CCC AGA GTG AAG GCT GTG
 I   Q   L   H          Intron 3                 G   P   P   R   V   K   A   V   110
AAG TCG TCAGAA CAC ATC AAC GAG GGG GAG ACG GCC ATG CTG GTC TGC AAG TCA GAG TCC
 K   S   S   E   H   I   N   E   G   E   T   A   M   L   V   C   K   S   E4S     130
GTG CCA CCT GTC ACT GAC TGG GCC TGG TAC AAG ATC ACT GAC TCT GAG GAC AAG Ggtgagaa
 V   P   P   V   I   D   W   A   W   Y   K   I   T   D   S   E   D   K           148
gcc—0.65 Kb—ttggccccctagCC CTC ATG AAC GGC TCC GAG AGC AGG TTC TTC GTG AGT TCC
   Intron 4                  A   L   M   N   G   S   E   S   F   F   V  5S   S   162
TCG CAG GGC CGG TCA GAG CTA CAC ATT GAG AAC CTG AAC ATG GAG GCC GAT CCC GGC CAG
 S   Q   G   R   S   E   L   H   I   E   N   L   N   M   E   A   D   P   G   Q   182
TAC CGG TGC AAC GGC ACC AGC TCC AAC GGC TCC GAC CAG GCC ATC ATC ACG CTC CGC GTG
 Y   R   C   N   G   T   S   S   K   G   S   D   Q   A   I   I   T   L   R   V   202
CGC AGC CAC CTG GCC GCC CTC TGG CCC TTC CTG GGC ATC GTG GCT GAG GTG CTG GTG CTG GTC
 R   S   H   L   A   A   L   W   P   F   L   G   I   V   A   E   V   L   V   L   V   223
ACC ATC ATC TTC ATC TAC GAG AAG CGC CGG AAG CCC GAG GAC GTC CTG GAT Ggtccttcttca
 T   I   I  6F   I   Y   E   K   R   R   K   P   E   D   V   L   D               240
—0.55 Kb—ggtccttcttcagAT GAC GAC GCC GGC TCT GCA CCC CTgtaagttcca—0.25 Kb—tcat
 Intron 5                  D   D   D   A   G   S   A   P   L       Intron 6      249
gcccagG AAG AGC AGC GGG CAG CAC CAG AAT GAC AAA GGC AAG AAC GTC CGC CAG AGG
         K   S   S   G   Q   H   Q   N   D   K   G   K7N   V   R   Q   R         266
AAC TCT TCC TGA GGCAGgtgcggcgtgg—0.3Kb—ccctgtccacagGTGGCCCGAG GACGCTCCCT
 N   S   S   *             Intron 7                                            269
GCTCCGCGTC TGCGCCGCCG CCGGAGTCCA CTCCCAGTGC TTGCAAGATT CCAAGTTCTC
                                  8
ACCTCTTAAA GAAAACCCAC CCCGTAGATT CCCATCATAC ACTTCCTTCT TTTTTAAAAA
AGTTGGGTTT TCTCCATTCA GGATTCTGTT CCTTAGGTTT TTTTCCTTCT GAAGTGTTTC
ACGAGAGCCC GGGAGCTGCT GCCCTGCGGC CCCGTCTGTG GCTTTCAGCC TCTGGGTCTG
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GCCCTTTGTG GAAAGTCACA GGTCACACGA GGGGCCCCGT GTCCTGCCTG TCTGAAGCCA
ATGCTGTCTG GTTGCGCCAT TTTTGTGCTT TTATGTTTAA TTTTATGAGG CCACGGGTCT
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ACACCGAGGG CGACCCCATC ACAGCCTCAA GTCACTCCCA AGCCCCCTCC TTGTCTATGC
ATCCGGGGGC AGCTCTGGAG GGGGTTTGCT GGGGAACTGG CGCCATCGCC GGGACTCCAG
AACCGCAGAA GCCTCCCCAG CTCACCCCTG GAGGACGGCC GGCTCTCTAT AGCACCAGGG
CTCACGTGGG AACCCCCCTC CCACCCACCG CCACAATAAA GATCGCCCCC ACCTCCA
                                            9
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Figure 3: Panel A. Probes used for S1 nuclease analysis. Solid black lines indicate vector portions of the probes. Numbering refers to Figure 2. Panel B. S1 nuclease analysis to determine the transcriptional start site. Lane 1, size marker--100 bp ladder; lane 2, size marker--ϕX 174 digested with Hae III; lane 3, the 331 bp EMMPRIN probe with some vector attached; lane 4, the vector /331 bp probe hybridized to mRNA, then digested with S1 nuclease; lane 5, the 492 bp probe with some vector attached; lane 6, the vector/492 bp probe hybridized to mRNA, then digested with S1 nuclease; lane 7, the negative control probe; lane 8 the negative control probe hybridized to mRNA, then digested with S1 nuclease; lane 9, the positive control probe derived from an internal region of the EMMPRIN cDNA; lane 10, the positive control probe hybridized to mRNA, then digested with S1 nuclease; lane 11, 100 bp ladder; lane 12, ϕX 174 digested with Hae III. Lanes 4 and 6 show that a 68 bp fragment of probes 1 and 2 is protected. Lane 8 shows that the negative control probe is not protected at all by the mRNA. Lane 10 shows that the 297 bp fragment from the interior of the cDNA is protected as expected.

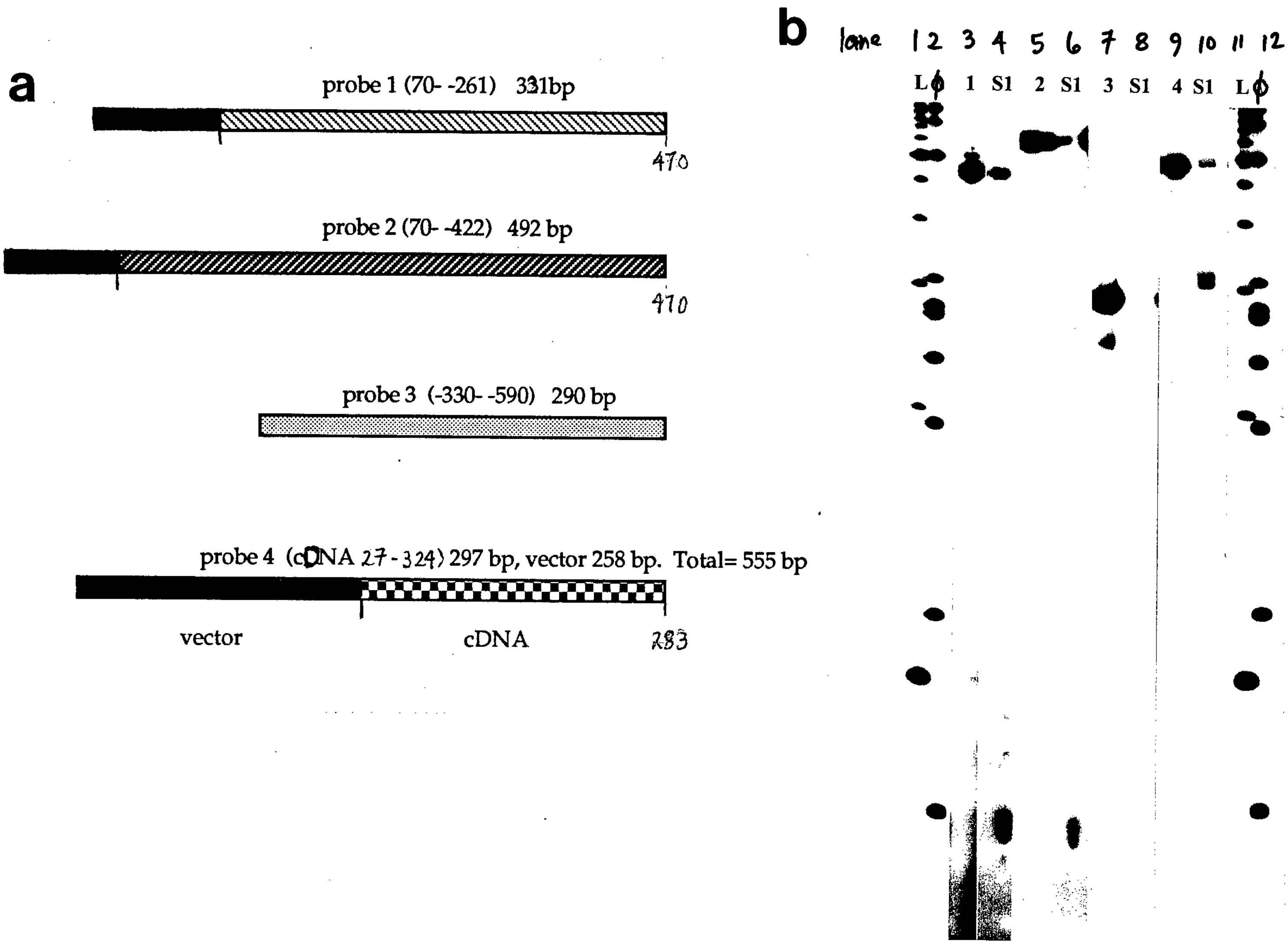


Figure 4: The EMMPRIN gene organization, outlining the exons and introns. The sum of the exon and intron sizes is 10.8 kb.

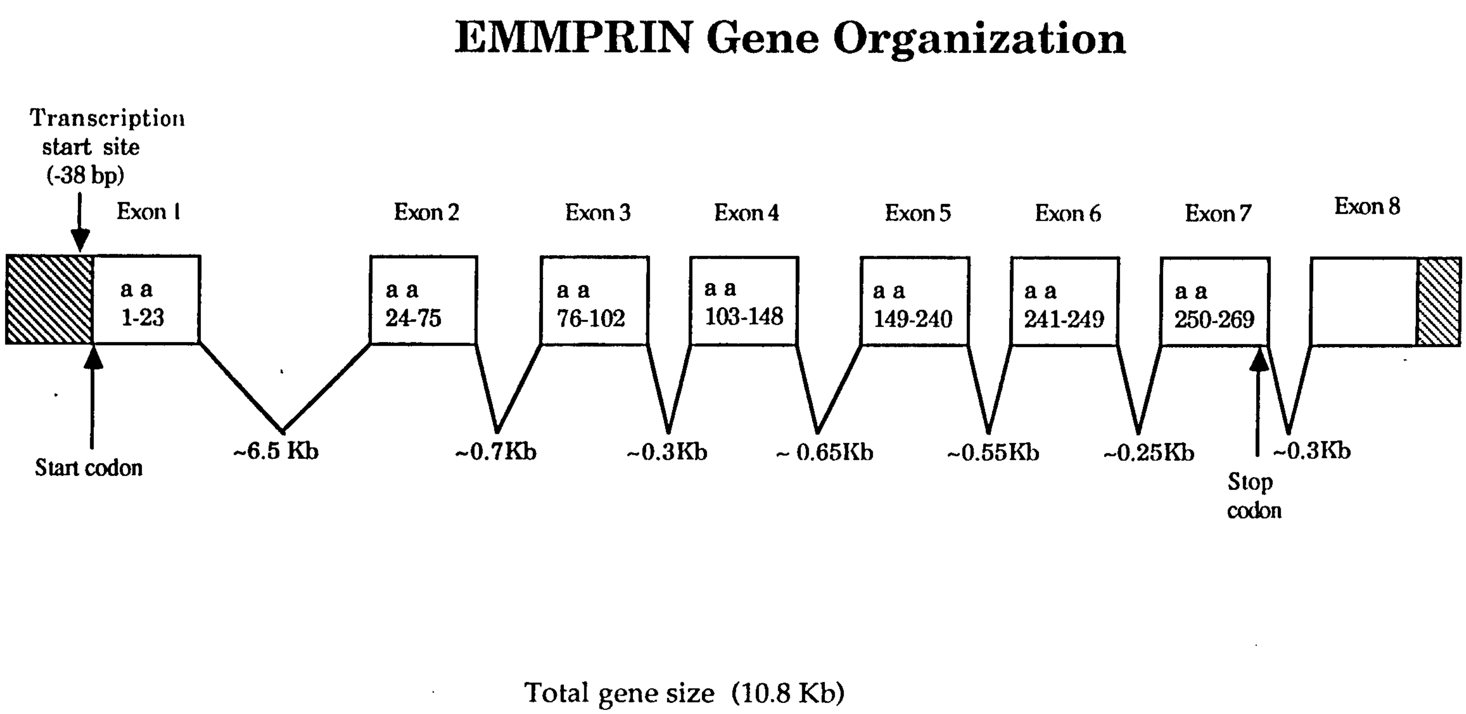


Figure 5: Schematic diagram of the mRNA, showing exon borders and domain structures. Starting from the left is the 5' UTR and the signal peptide encoded by Exon 1. The two half circles indicate the Ig domains of the extracellular portion of the molecule, showing that each is encoded by two exons. The transmembrane domain is above the cross-hatched gray structure labeled "membrane". To the right of the transmembrane domain are the 2 exons encoding the cytoplasmic domain and the 1 exon (exon 8) encoding the 3' UTR.

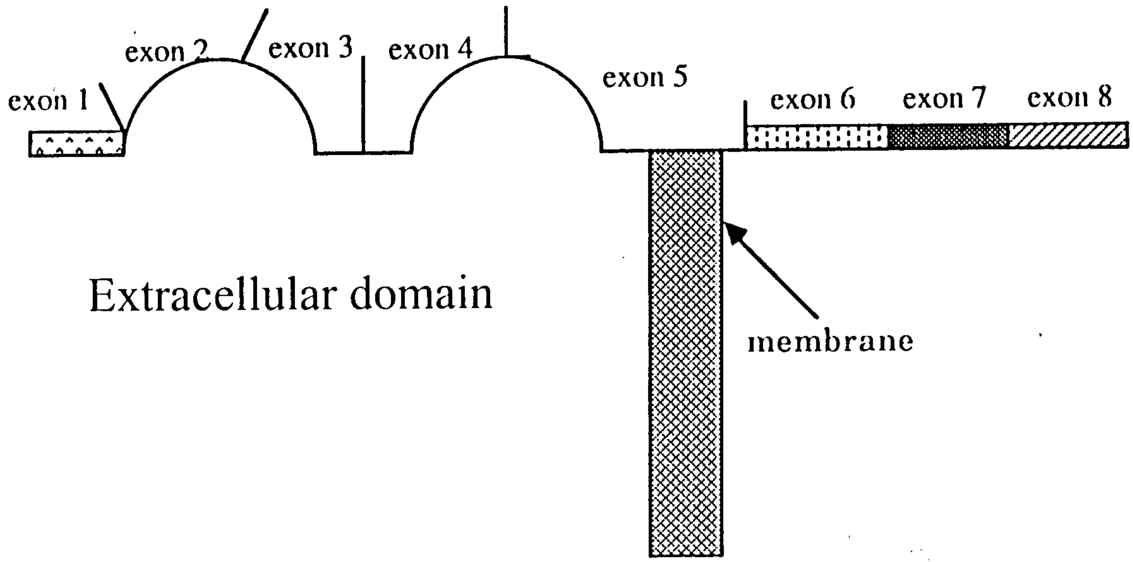


Figure 6: Schematic of the 5' flanking regions of the human and mouse EMMPRIN genes. +1 indicates the transcription start site. The dashed line symbolizes the CpG island. Filled in circles denote SP1 binding sites and filled in boxes denote AP2 binding sites. The small A/T regions that may facilitate RNA polymerase binding are indicated by unfilled triangles. The ATG start codes are marked.

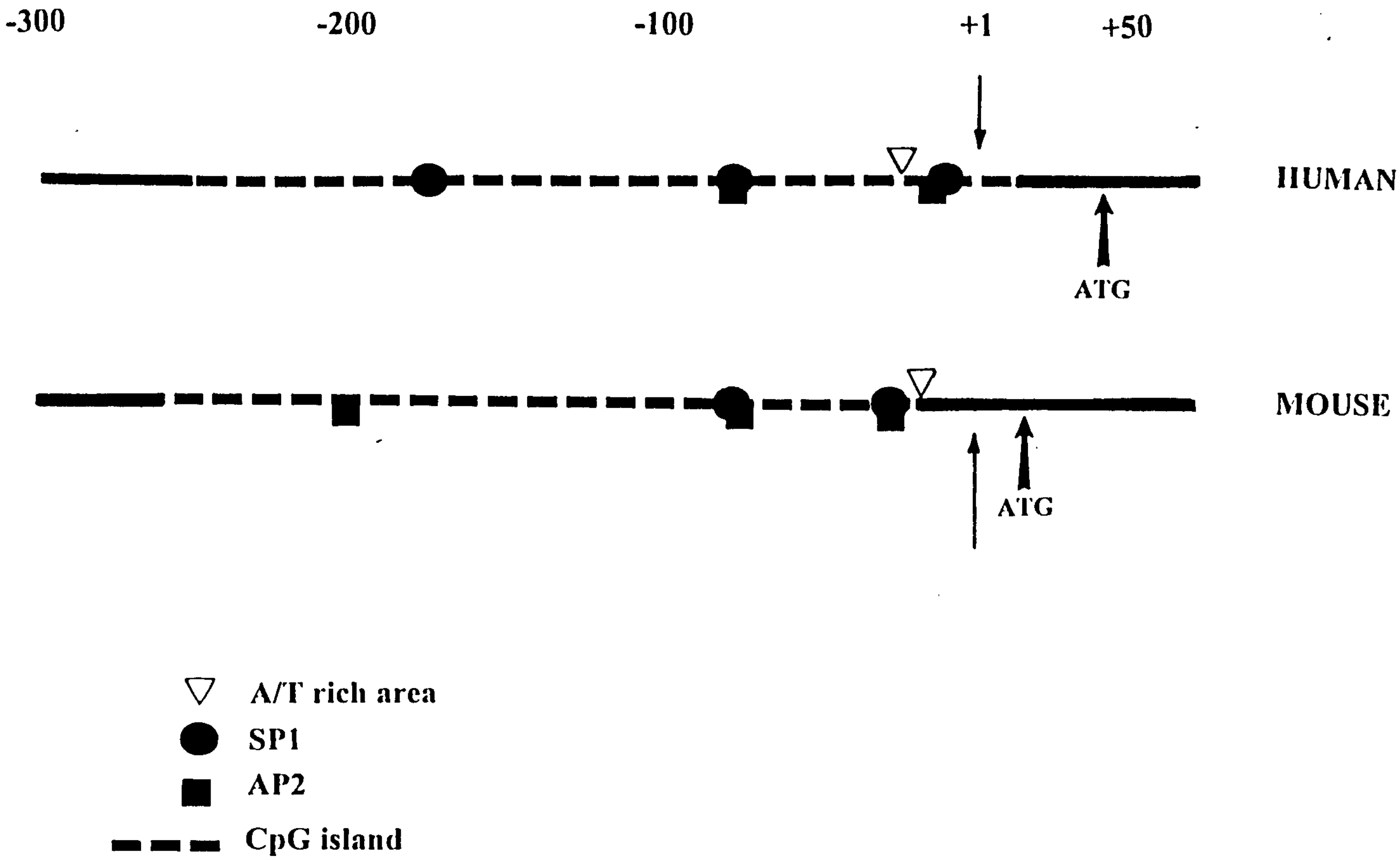


Figure 7: Northern blot analysis of total RNA extracted from MDA-MB-436 human breast cancer cells transfected with EMMPRIN cDNA. Ten ug of total RNA was loaded and probed with nick-translated, radiolabeled cDNA(TALT5j). Total radioactivity used was 1 X 10^6 cpm/ml, and the duration of film exposure was 18 hours (1). Lane 1, cells transfected with EMMPRIN cDNA.; lane 2, cells transfected with vector cDNA only; lane 3, EMMPRIN mRNA isolated from transfected CHO cells. EMMPRIN mRNA is identified at 1.4 kb. Total RNA content of each lane was comparable (data not shown).

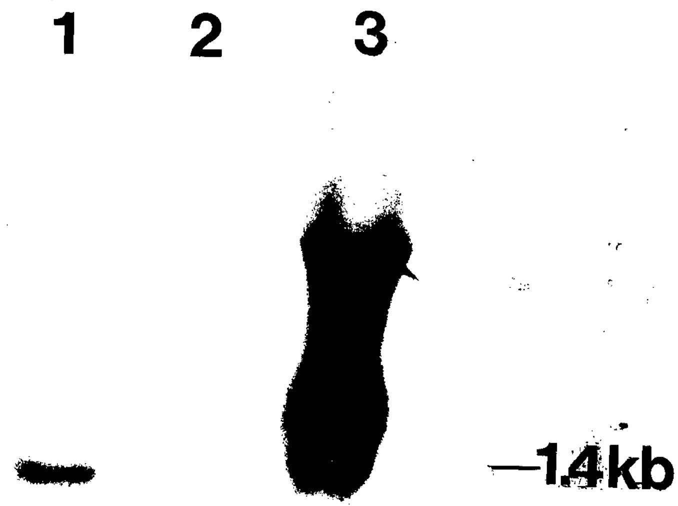


Figure 8: Nude mice injected with MDA-MB-436 breast cancer cells transfected with EMMPRIN/GFP cDNA develop tumors more rapidly than mice transfected with GFP cDNA alone. Figure A: The mouse on the left was injected with GFP transfected MDA-MB-436 cells and the mouse on the mouse right was transfected with EMMPRIN/GFP transfected cells. Nude mice were sacrificed 12 weeks after injection with 1 X 10^7 cells into the inferior mammary fat pad. Figure B depicts the growth rate of these tumors in mice as measured with a caliper at weekly intervals.

A

B

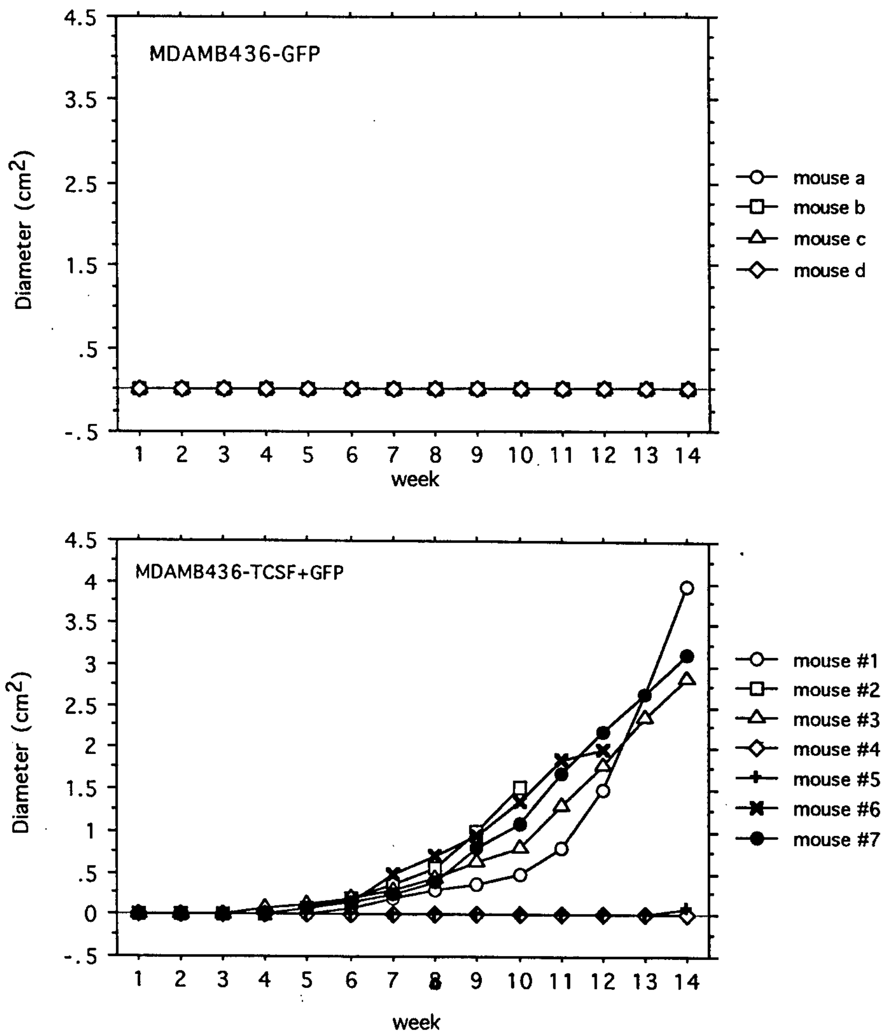


Figure 9- Demonstration of a receptor for EMMPRIN on the surface of intact fibroblasts using a cross-linking reagent. Lanes 1 and 2 contain EMMPRIN protein cross linked (DTSSP) with ^{35}S-labeled fibroblasts; lane 3 shows the result in the absence of the cross linking reagent (DTSSP); lane 4 shows the cross linking reaction with EMMPRIN omitted.

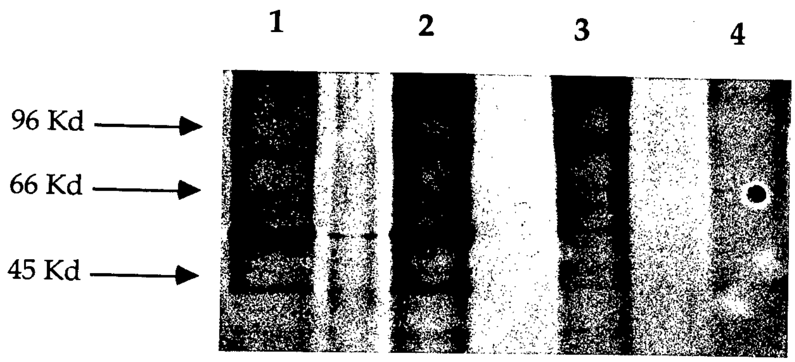


Figure 10- Binding of solubilized fibroblast membrane proteins to immobilized EMMPRIN. Confirmation of an EMMPRIN receptor on fibroblasts. Lane 1 contains the first elution from the Carbolink column; lane 2 contains the second elution from the Carbolink column; lane 3 contains the elution from the Aminolink column.

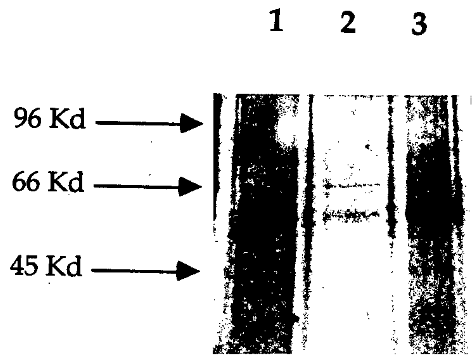
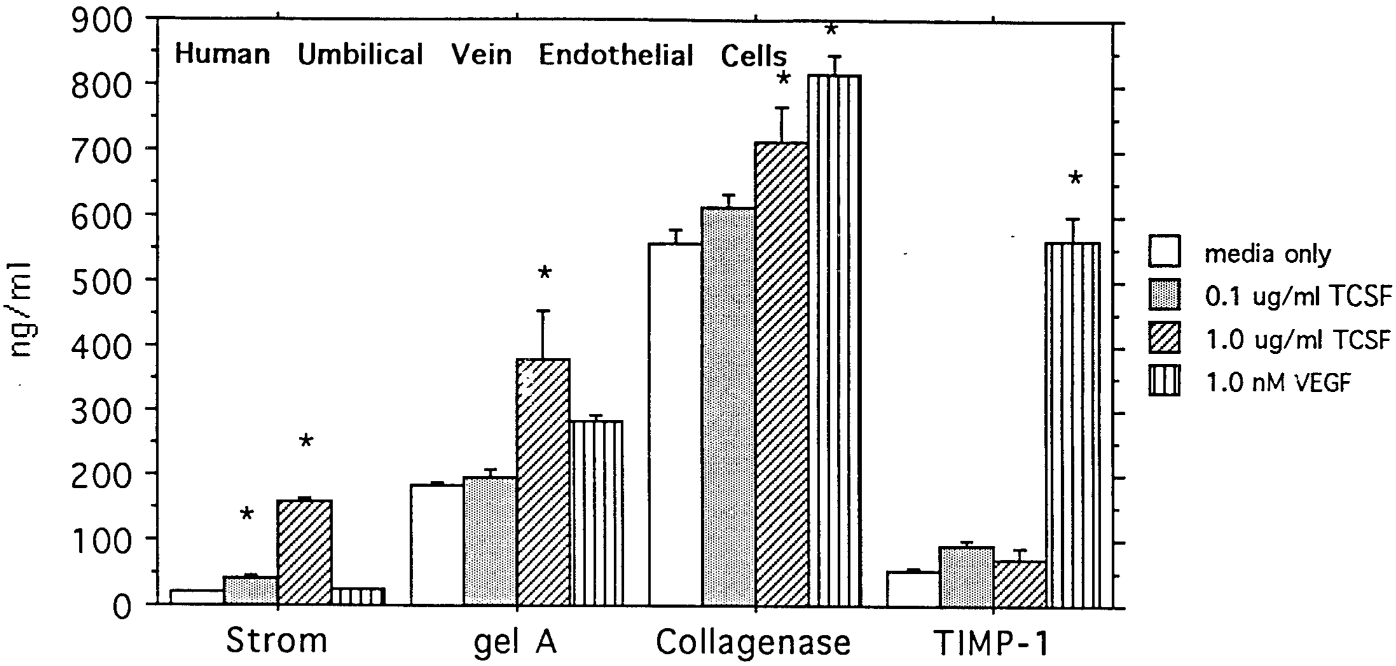


Figure 11: EMMPRIN induces endothelial cells to produce stromelysin-1, gelatinase A, and interstitial collagenase; vascular endothelial growth factor (VEGF) induces synthesis of interstitial collagenase and TIMP-1. Human umbilical vein endothelial cells were incubated with EMMPRIN, VEGF, or vehicle for 18 hours in serum-free media. Conditioned media was collected and tested for stromelysin-1, gelatinase A, and TIMP-1 employing specific ELISAs as we previously demonstrated (27). Synthesis of all three proteins was demonstrated.



Stimulation of MMP and TIMP by EMMPRIN and VEGF
(18 h. incubation)

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1. Biswas, C., Zhang, Y., DeCastro, R., Guuo, H., Nakamura, T., Kataoka, H. and Nabeshima, K. The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. Cancer Res., *55*: 434-439, 1995.
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The Human Tumor Cell-derived Collagenase Stimulatory Factor (Renamed EMMPRIN) Is a Member of the Immunoglobulin Superfamily[1]

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ABSTRACT

Tumor cell-derived collagenase stimulatory factor, renamed extracellular matrix metalloproteinase inducer (EMMPRIN), is a M_r ~58,000 glycoprotein which is located on the outer surface of human tumor cells and which interacts with fibroblasts to stimulate expression of several matrix metalloproteinases in the fibroblasts. In this study, we have used several approaches to isolate a complementary DNA encoding EMMPRIN. Several peptide sequences obtained from the isolated M_r 58,000 glycoprotein are found in the translated complementary DNA clone, verifying its identity. Computer database searches indicate that EMMPRIN is a member of the immunoglobulin superfamily and that the deduced amino acid sequence of EMMPRIN is identical to that recently reported for human basigin and M6 antigen, molecules of previously undetermined biological function.

INTRODUCTION

Degradation of extracellular matrix components of the basement membrane and interstitial matrix by MMPs[6] is a crucial step in tumor cell invasion and metastasis (1–3). The role of tumor cell-fibroblast interactions in regulation of MMP levels in neoplasms has been demonstrated by several investigators, including ourselves (4–7). The recent finding (8–11) that, *in vivo*, some tumor-associated MMPs are mainly synthesized in peritumoral fibroblasts, rather than in tumor cells themselves, is consistent with a major role for these interactions in tumorigenesis *in vivo*.

We have shown that tumor cells in culture stimulate fibroblasts to produce high levels of collagenase and that a factor (previously termed TCSF) that is associated with tumor cell membranes, but also released into medium conditioned by tumor cells, is responsible for this stimulation (6, 12, 13). We have immunoaffinity purified the M_r ~58,000 TCSF from a human lung carcinoma cell line, LX-1 (13, 14), and demonstrated that addition of this purified factor to cultured fibroblasts stimulates expression, not only of interstitial collagenase (MMP-1), but also of fibroblast-derived stromelysin-1 (MMP-3) and M_r 72,000 gelatinase (MMP-2) (15, 16). While immunohistochemical studies have shown that TCSF is highly enriched around the outer surface of tumor cells and absent from most normal cells *in vivo* (17), recent studies from our laboratory[7] have shown that TCSF is also present on the surface of normal human keratinocytes, where it presumably plays a role in regulating stromal MMPs (18). For these reasons, we have now renamed this factor EMMPRIN to indicate its role in extracellular matrix metalloproteinase induction via normal, as well as pathological, cellular interactions.

To help understand the chemical and biological nature of EMMPRIN and its relationship to other proteins, we have attempted to isolate cDNA clones for the protein. Oligonucleotide primers derived from peptide sequences were used to isolate EMMPRIN cDNAs by RT-PCR. Analysis of the cDNA-derived amino acid sequence of EMMPRIN indicates that it is a member of the immunoglobulin superfamily. Interestingly, the sequence is identical to two recently reported human cDNAs of unknown function, *i.e.*, human basigin (19) and M6 antigen (20). Thus, our studies provide one important function for these proteins, namely intercellular stimulation of MMP synthesis.

MATERIALS AND METHODS

Amino Acid Sequencing. Previously, we have reported the amino acid sequences for the NH_2-terminus of EMMPRIN and four peptides derived from EMMPRIN after trypsin digestion (14). We have now sequenced two more peptides derived from EMMPRIN in the same manner as described previously (14). Briefly, immunopurified EMMPRIN was subjected to SDS-PAGE and blotted to a nitrocellulose membrane. The EMMPRIN band was revealed by staining with Ponceau S. After destaining, the protein band was cut from the membrane and digested with trypsin at a ratio of 1:20 (w/w). The peptides were separated by reverse phase HPLC, and the samples were subjected to automated Edman degradation.

cDNA Synthesis. RNA was prepared from LX-1 cells by a routine procedure using guanidinium thiocyanate (21), and poly(A)$^+$RNA was isolated using the Mini Ribosep mRNA isolation kit according to the manufacturer's instructions (Collaborative Biomedical Products, Bedford, MA). First-strand cDNA was synthesized by reverse transcription of 1 μg of poly(A)$^+$ RNA using Moloney murine leukemia virus reverse transcriptase, according to the manufacturer's instructions (GIBCO-BRL, Gaithersburg, MD) in the presence of random hexamers or specific primers, depending on the desired reaction product. The resulting reaction mixture was digested with RNase H and used as a template for PCR.

DNA Amplification. PCR-amplified DNA fragments were generated with a Perkin-Elmer Cetus DNA thermal cycler (Norwalk, CT) using a gene amplification kit (Perkin Elmer) according to the manufacturer's instructions. Briefly, 100 μl of reaction mixture contained 100 ng of cDNA pool, 10 μl of 10X PCR buffer (provided in the kit), 16 μl of each deoxynucleotide triphosphate at 1.25 mM, 5 μl each of 20 μM primers, and 0.5 μl of Taq-DNA polymerase (2.5 units per assay). Samples were subjected to 30 cycles at the following conditions: 1 min at 94°C for denaturation; 1 min at 48°C for annealing; and 1.5 min at 72°C for elongation. A final elongation step consisted of a 72°C incubation for 10 min. Amplified products were separated by agarose gel electrophoresis and were identified by ethidium bromide staining. Where reamplification of a PCR product was necessary, samples were applied to low-melting point agarose gels (FMC, Rockland, ME); bands were excised, melted, and purified on Spin Bind extraction cartridges (FMC).

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[2] This article is dedicated to the memory of Dr. Chitra Biswas, who passed away August 26, 1993. Requests for reprints should be addressed to Dr. Bryan Toole at Department of Anatomy and Cellular Biology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111.

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[6] The abbreviations used are: MMP, matrix metalloproteinase; EMMPRIN, extracellular matrix metalloproteinase inducer; PCR, polymerase chain reaction, RT-PCR, reverse transcriptase-PCR; TCSF, tumor cell-derived collagenase stimulatory factor; bp, base pair(s); kb, kilobase.

[7] R. DeCastro, Y. Zhang, H. Guo, H. Kataoka, and C. Biswas. Human keratinocytes express EMMPRIN, an extracellular matrix metalloproteinase inducer, manuscript in preparation.

Generation of an Authentic cDNA for EMMPRIN Peptide #51. Two 17-mer, degenerate, oligonucleotide mixtures, based on the previously obtained amino acid sequence of EMMPRIN peptide #51 (14), were synthesized. One of these, termed A, was synthesized in the sense direction corresponding to amino acids 2–7; the other, termed C′, was synthesized in the antisense direction corresponding to amino acids 13–18. The sequences of the oligonucleotide mixtures are shown in Table 1. These mixtures were used as primers in RT-PCR to generate an authentic EMMPRIN cDNA corresponding to amino acid residues 2–18 of peptide #51, using LX-1 cell mRNA as the initial template. The primers had 8-bp adapters with restriction site sequences (*Eco*RI for the 5′-end of A and *Pst*I for the 5′-end of C′; not shown in Table 1), allowing subsequent cloning of the product in the event that difficulty was encountered in directly ligating the product into the pCRII vector. The resulting PCR products were sized by electrophoresis against known DNA markers in 6% agarose (NuSieve GTG low melting point agarose; FMC). Several products of different intensities were identified, including the expected 66-bp product corresponding to #51 (50-bp fragment plus 2 × 8 bp adapters at 5′ and 3′ ends). The band corresponding to this size was cut out from the agarose gel and reamplified using the same primers A and C′ as described above. After checking the size of the reamplified product, all of which was 66 bp, the reaction mixture was used directly for ligating into the pCRII vector system (Invitrogen). Recombinants were selected as white colonies on plates containing 5-bromo-4-chloro-3-indolyl-*b*-D-galactoside. Plasmid DNA was isolated from seven of these colonies, and the sizes of the inserts were analyzed by PCR amplification using M13 primers (forward and reverse), followed by agarose gel electrophoresis.

All seven recombinants had inserts of the expected sizes. DNA from two of these clones was sequenced by the dideoxy-mediated chain termination method (22) using a double-stranded DNA cycle sequencing kit (GIBCO-BRL). The nucleotide sequence of the inserts from both clones exactly matched the amino acid sequence of peptide #51 (Fig. 1). However, the nucleotide sequence varied at three positions, all within the primer sequences, as shown in *bold* in Fig. 1. This suggests that several of the degenerate primers were used by the template cDNA during amplification. The use of degenerate oligonucleotide mixtures as primers often leads to variations of this kind in the regions of the cDNA that correspond to the primers (23). To avoid these regions of variation, we designed primers (B/B′) based on the sequence of the central part of the cDNA (Fig. 1) for use in the overlap extension reactions described below.

Generation of cDNAs Corresponding to the 5′ and 3′ Regions of EMMPRIN. To obtain a cDNA that includes the region of EMMPRIN that is 5′ to peptide #51, primer B′ (designed as described above) was used in the PCR in combination with primer D, a degenerate mixture corresponding to part of the previously sequenced NH_2-terminal peptide #59, derived from EMMPRIN (Ref. 14, see Table 1). The PCR products were directly ligated into pCRII (Invitrogen) and used to transform *Escherichia coli.* Insert-containing white colonies were selected, and the plasmid DNAs were isolated, sized, and sequenced. One of these cDNAs, TALT5j, was used for further study.

To obtain a cDNA that includes the region of EMMPRIN that is 3′ to peptide #51, we used the rapid amplification of cDNA ends protocol as described previously (24). A pool of cDNA was prepared from poly(A)⁺ RNA of LX-1 cells using the dT_{17} adapter primer E of 5′-GAATTCGAATTCGATATCTTTTTTTTTTTTTTTTT. The reaction mixture was then amplified in PCR using the dT_{17} adapter primer E and primer B, derived from peptide #51 (Table 1). After ligating the PCR products into the pCRII vector, transforming *E. coli*, and selecting white colonies, the plasmid DNA was isolated, and the inserts were sized and sequenced. One of these cDNAs, TALT3g, was used for further study.

Table 1 *EMMPRIN peptide sequences and derived oligonucleotide primers*

The amino acid sequences of the peptides for which corresponding oligonucleotide primers were synthesized are underlined. Oligonucleotide sequences A, B, and D are shown in the sense orientation, whereas B′ and C′ are in the antisense orientation. Primers A, C′, and D are degenerate mixtures; primers B and B′ are specific sequences derived as described in the text and Fig. 1. The nucleotide sequence, GARGA, highlighted in bold print at the 3′ end of primer D fortuitously matches a portion of the 5′ untranslated region of EMMPRIN (with the sequence, GAGGA) and thus amplified a cDNA beginning at this position (see Fig. 2).

	Peptide sequence		Oligonucleotide primer[a]
#51	SELHIENLNMEADPGQYR	A:	5′-GAR-YTN-CAY-ATM-GAR-AA-3′
	SELHIENLNMEADPGQYR	B:	5′-AAC-CTG-AAC-ATG-GAG-GCC-GA-3′
	SELHIENLNMEADPGQYR	B′:	5′-TC-GGC-CTC-CAT-GTT-CAG-GTT-3′
	SELHIENLNMEADPGOYR	C′:	5′-CZ-RTA-YTG-NCC-NGG-RTC-3′
#59	AAGTVFTTVEDLGSK	D:	5′-TTY-ACN-ACN-GTN-**GAR-GA**-3′

[a] M = A, C, or T; N = A, C, G, or T; Y = T or C; R = A or G; Z = G or T.

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-------Primer A------>                              <-----Primer C'-------

GAA-CTT-CAC-ATT-GAG-AAC-CTG-AAC-ATG-GAG-GCC-GAT-CCC-GGC-CAA-TAC-CG

-E---L---H---I---E---N---L---N---M---E---A---D---P---G---Q---Y---R-
```

Fig. 1. Sequence of authentic cDNA for EMMPRIN peptide #51. The positions of primers A and C′ used to obtain the authentic cDNA for peptide #51 are shown on the *top line*; the nucleotide sequence of one of the cDNAs obtained from PCR is given on the *second line*; the amino acid sequence of peptide #51 (residues #2–18) is on the *third line*. The amino acid sequence deduced from the nucleotide sequence is identical to that obtained by amino acid sequencing. A second clone was sequenced, and differences were found in the positions highlighted in *bold print* at the third, sixth and forty-fifth positions of the nucleotide sequence; all of these lie within regions corresponding to the primers, and this variation was presumably due to the use of degenerate oligonucleotides as primers. Primers B (*underlined*) and B′ were designed from the central part of this cDNA.

Construction of a cDNA Encoding the Complete EMMPRIN Sequence. The inserts of the 5′ and 3′ cDNA clones for EMMPRIN (TALT5j and TALT3g) overlapped by 20 nucleotides and thus were used in the PCR-based, overlap extension technique (25) to yield a single cDNA with the complete open reading frame for EMMPRIN.

First, the cDNAs corresponding to the 5′ region (TALT5j) and the 3′ region (TALT3g) were amplified by PCR in two separate reactions. For TALT5j, a 30-mer sense primer, composed of a 13-bp *Bam*HI adapter at the 5′ end followed by a 17-mer corresponding to the 5′ terminus of TALT5j, was used (primer F, 5′-CGCGGATCCCGGCGAGGAATAGGAATCATG); the antisense primer was a 20-mer which corresponded to the 3′ terminus of TALT5j (primer B′ in Table 1). For TALT3g, a 20-mer sense primer corresponding to the 5′ terminus of TALT3g (primer B in Table 1) and the dT_{17} adapter primer E (5′-GAATTCGAATTCGATATCTTTTTTTTTTTTTTTTT) were used. The amplified products were electrophoresed in agarose, and the bands corresponding to 0.6 kb (TALT5j) or 1.1 kb (TALT3g) were identified by ethidium bromide staining and excised.

In the third and final reaction, the gel-purified products from both the above reactions were used as templates for fusion by overlap extension (25). For this reaction, the 30-mer primer F (5′-most primer), used above for amplification of TALT5j, was used with the dT_{17} adapter primer E (3′-most primer). The 1.6-kb DNA product that was generated in this PCR was ethanol precipitated, agarose gel-purified, and subcloned into pBluescript. Sequencing of this cDNA confirmed its identity with the combined sequence of the two separate cDNAs. The complete sequence is shown in Fig. 2.

Northern Blot Analysis. Northern blot analysis was performed as described previously (16). Ten μg of total RNA was electrophoresed on a 1% agarose gel and transferred to nitrocellulose membrane. The blot was then hybridized overnight with EMMPRIN cDNA, which had been radiolabeled by nick-translation with ^{32}P-labeled dCTP. After washing, the filter was exposed to Kodak XR-5 film at −80°C for 48 h.

Deletion Analyses. The strategy used for making deletion constructs was adapted from that used to create the full-length EMMPRIN cDNA, *i.e.*, overlap extension (25). The primers used for the following reactions were primer F, 5′-CGCGGATCCCGGCGAGGAATAGGAATCATG-3′; primer G, 5′-ACGGAGCCTCCGGGGTGAAGGCTGTGAAGTCG-3′; primer H, 5′-ACGGGCCTCCCAGAAGCCACCTGGCCGCCCTC-3′; primer I, 5′-GCGTGCGCAGCCACGAGAAGCGCCGGAAGCCC-3′; and primer J, 5′-TTCATCTACTAGTAGCGCCGGAA-3′.

The extracellular immunoglobulin domain I deletion construct was made as follows: (*a*) two PCRs, using EMMPRIN cDNA as template, were used to synthesize DNA fragments on each side of the desired deletion, one with the primers F (a *Bam*HI adapter plus the 5′ end of the EMMPRIN cDNA sequence) and reverse complement of G, and the other with primer G and the dT_{17} adapter primer E. Primer G is composed of sequences from each side of the desired deletion, *i.e.*, nucleotides 50–63 linked directly to 319–336 (Fig. 2). PCR products were analyzed and purified on 1% low-melting point agarose. DNA of expected sizes, 108 bp from the first reaction and 1306 bp from the second reaction, were cut from the gels and incubated in 300 μl H_2O at

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-14              +1
 GAGGAATAGGAATC ATG GCG GCT GCG CTG TTC GTG CTG CTG GGA TTC GCG CTG CTG GGC ACC CAC GGA GCC TCC GGG GCT GCC GGC
                 M   A   A   A   L   F   V   L   L   G   F   A   L   L   G   T   H   G   A   S   G   A   A   G   24

  73 ACA GTC TTC ACT ACC GTA GAA GAC CTT GGC TCC AAG ATA CTC CTC ACC TGC TCC TTG AAT GAC AGC GCC ACA GAG GTC ACA
      T   V   F   T   T   V   E   D   L   G   S   K   I   L   L   T   C   S   L   N   D   S   A   T   E   V   T   51
              Peptide 59
 154 GGG CAC CGC TGG CTG AAG GGG GGC GTG GTG CTG AAG GAG GAC GCG CTG CCC GGC CAG AAA ACG GAG TTC AAG GTG GAC TCC
      G   H   R   W   L   K   G   G   V   V   L   K   E   D   A   L   P   G   Q   K   T   E   F   K   V   D   S   78

 235 GAC GAC CAG TGG GGA GAG TAC TCC TGC GTC TTC CTC CCC GAG CCC ATG GGC ACG GCC AAC ATC CAG CTC CAC GGG CCT CCC
      D   D   Q   W   G   E   Y   S   C   V   F   L   P   E   P   M   G   T   A   N   I   Q   L   H   G   P   P  105
                 Peptide 74
 316 AGA GTG AAG GCT GTG AAG TCG TCA GAA CAC ATC AAC GAG GGG GAG ACG GCC ATG CTG GTC TGC AAG TCA GAG TCC GTG CCA
      R   V   K   A   V   K   S   S   E   H   I   N   E   G   E   T   A   M   L   V   C   K   S   E   S   V   P  132
                                                                                                 Peptide 61
 397 CCT GTC ACT GAC TGG GCC TGG TAC AAG ATC ACT GAC TCT GAG GAC AAG GCC CTC ATG AAC GGC TCC GAG AGC AGG TTC TTC
      P   V   T   D   W   A   W   Y   K   I   T   D   S   E   D   K   A   L   M   N   G   S   E   S   R   F   F  159

 478 GTG AGT TCC TCG CAG GGC CGG TCA GAG CTA CAC ATT GAG AAC CTG AAC ATG GAG GCC GAT CCC GGC CAG TAC CGG TGC AAC
      V   S   S   S   Q   G   R   S   E   L   H   I   E   N   L   N   M   E   A   D   P   G   Q   Y   R   C   N  186
        Peptide 26                                           Peptide 51
 559 GGC ACC AGC TCC AAG GGC TCC GAC CAG GCC ATC ATC ACG CTC CGC GTG CGC AGC CAC CTG GCC GCC CTC TGG CCC TTC CTG
      G   T   S   S   K   G   S   D   Q   A   I   I   T   L   R   V   R   S   H   L   A   A   L   W   P   F   L  213

 640 GGC ATC GTG GCT GAG GTG CTG GTG CTG GTC ACC ATC ATC TTC ATC TAC GAG AAG CGC CGG AAG CCC GAG GAC GTC CTG GAT
      G   I   V   A   E   V   L   V   L   V   T   I   I   F   I   Y   E   K   R   R   K   P   E   D   V   L   D  240

 721 GAT GAC GAC GCC GGC TCT GCA CCC CTG AAG AGC AGC GGG CAG CAC CAG AAT GAC AAA GGC AAG AAC GTC CGC CAG AGG AAC
      D   D   D   A   G   S   A   P   L   K   S   S   G   Q   H   Q   N   D   K   G   K   N   V   R   Q   R   N  267
      Peptide 38
 802 TCT TCC TGA GGCAGGTGGC  CCGAGGACGC  TCCCTGCTCC  GCGTCTGCGC  CGCCGCCGGA  GTCCACTCCC  AGTGCTTGCA  AGATTCCAAG
      S   S   *

 891 TTCTCACCTC  TTAAAGAAAA  CCCACCCCGT  AGATTCCCAT  CATACACTTC  CTTCTTTTTT  AAAAAAGTTG  GGTTTTCTCC  ATTCAGGATT

 981 CTGTTCCTTA  GGTTTTTTTC  CTTCTGAAGT  GTTTCACGAG  AGCCCGGGAG  CTGCTGCCCT  GCGGCCCCGT  CTGTGGCTTT  CAGCCTCTGG

1071 GTCTGAGTCA  TGGCCGGGTG  GGCGGCACAG  CCTTCTCCAC  TGGCCGGAGT  CAGTGCCAGG  TCCTTGCCCT  TTGTGGAAAG  TCACAGGTCA

1161 CACGAGGGGC  CCCGTGTCCT  GCCTGTCTGA  AGCCAATGCT  GTCTGGTTGC  GCCATTTTTG  TGCTTTTATG  TTTAATTTTA  TGAGGGCCAC

1251 GGGTCTGTGT  TCGACTCAGC  CTCAGGGACG  ACTCTGACCT  CTTGGCCACA  GAGGACTCAC  TTGCCCACAC  CGAGGGCGAC  CCCATCACAG

1341 CCTCAAGTCA  CTCCCAAGCC  CCCTCCTTGT  CTATGCATCC  GGGGGCAGCT  CTGGAGGGGG  TTTGCTGGGG  AACTGGCGCC  ATCGCCGGGA

1431 CTCCAGAACC  GCAGAAGCCT  CCCCAGCTCA  CCCCTGGAGG  ACGGCCGGCT  CTCTATAGCA  CCAGGGCTCA  CGTGGGAACC  CCCCTCCCAC

1521 CCACCGCCAC  AATAAAGATC  GCCCCCACCT  CCAAAAAAAA  AAAAAAAAAA  AAAAAAAAAA  AAAAAAAAAA  AA
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Fig. 2. Sequence of human EMMPRIN cDNA.[8] Nucleotide sequence and derived amino acid sequence for human EMMPRIN; nucleotides are numbered on the *left* and amino acids on the *right*. The putative signal and transmembrane amino acid sequences and the polyadenylation signal are *underlined*. The *shaded regions* indicate the deduced amino acid sequences that match the six peptides derived from EMMPRIN. The 20-nucleotide overlapping region of the 5′ and 3′ cDNAs, TALT5j and TALT3g, is indicated by a *line* above nucleotide positions #517–536. The stop codon is marked by an *asterisk*.

65°C for 10 min; (*b*) a PCR was performed with 10 ng each of these two fragments, plus primer F and the dT_{17} adapter. The PCR product was precipitated with 0.3 M ammonium acetate and 2 volumes of ethanol, *Eco*RI, and electrophoresed on 1% low-melting point agarose. DNA with the expected size, 1382 bp, was cut from the gel and purified on Spin bind extraction cartridge; and (*c*) the purified DNA was ligated into pBluescript, and used to transform XL-1 blue cells. Plasmid DNA was isolated from positive (white colony) transformants and confirmed by sequencing.

To create constructs with deletions in the extracellular immunoglobulin domain II, the transmembrane domain, and the cytoplasmic domain, the same approach was used with appropriate primer pairs. For deleting the extracellular immunoglobulin domain II, primers F and the reverse complement of H, and primer H and the dT_{17} adapter, were used to make fragments on each side of the desired deletion. Primer H contains sequences from both sides of the region we wished to delete, bases 305–318 linked directly to 610–627 (Fig. 2). To delete the transmembrane domain, the two fragments were made with primers F and the reverse complement of I, and primer I with the dT_{17} adaptor. Primer I consists of bases 602–615 linked to 688–705 (Fig. 2), sequences on each side of the desired deletion. Primer F and the dT_{17} adapter were used in the final PCR to make the fused deletion product from the two fragments. To delete the cytoplasmic domain, two premature stop codons were inserted into the EMMPRIN cDNA. PCR fragments were made from template cDNA using primers F and the reverse complement of J, and J with the dT_{17} adapter. Primer J corresponds to nucleotides 679–701 with T residues substituted for G and A at positions 688 and 691, respectively (Fig. 2) to create new termination codons. The two fragments were fused by overlap extension with primer F and the dT_{17} adapter. These deletion constructs were also subcloned into pBluescript and used to transform XL-1 blue cells.

To express each deletion construct, 20 μl of overnight cultures of each bacterial stock were added to 2 ml of Luria broth containing 0.2% glucose and 50 μg/ml ampicillin. After incubating at 37°C for 2 h, the cells were further incubated in the absence or presence of 0.5 mM isopropylthio-*b*-D-galactoside at 37°C for 3 h. The cells were collected by centrifugation and extracted by 30 μl of 2 × SDS-PAGE sample buffer. The cell extracts were separated on 15% SDS-PAGE and then analyzed for cross-reactivity with the monoclonal antibody E11F4 by Western blotting (26).

RESULTS

Amino Acid Sequences of Peptides Derived from EMMPRIN. Previously, we have reported the amino acid sequence of four peptides termed #26, #51, #59, and #61, obtained by HPLC fractionation of tryptic peptide digests of EMMPRIN (14). Subsequently, we have sequenced two additional peptides, #38 and #74, from EMMPRIN. The sequences of the six peptides are shown in Table 2.

Isolation of EMMPRIN cDNAs. Several attempts to obtain EMMPRIN cDNA clones by screening an LX-1 library with degenerate or best-guess oligonucleotide probes, derived from the peptide sequences, were unsuccessful. Therefore, the following steps were taken to obtain a cDNA for EMMPRIN. First, a small EMMPRIN-specific cDNA corresponding to a single peptide-derived sequence, peptide #51, was generated by RT-PCR with degenerate primers. Isolation of this cDNA confirmed the presence of an mRNA contain-

Table 2 *Amino acid sequences of peptides derived from EMMPRIN*

Peptides #38 and #74 (marked with an asterisk) are reported here for the first time; peptides #26, #51, #59, and #61 were reported previously (14).

Peptide #26:	Phe-Phe-Val-Ser-Ser-Ser-Gln-Gly-Arg
Peptide #38*:	Pro-Glu-Asp-Val-Leu-Asp-Asp-Asp-Asp-Ala-Gly-Ser-
Peptide #51:	Ser-Glu-Leu-His-Ile-Glu-Asn-Leu-Asn-Met-Glu-Ala-Asp-Pro-Gly-Gln-Tyr-Arg
Peptide #59:	Ala-Ala-Gly-Thr-Val-Phe-Thr-Thr-Val-Glu-Asp-Leu-Gly-Ser-Lys
Peptide #61:	Ser-Glu-Ser-Val-Pro-Pro-Val-Thr-Asp
Peptide #74*:	Val-Asp-Ser-Asp-Asp-Gln-Trp-Gly-Glu-Tyr-Ser-X-Val-Phe-Leu-Pro-Glu-

[8] The sequence reported in this publication has been deposited in the GenBank data base (accession no. L10240).

ing the EMMPRIN-derived sequence in LX-1 cells and provided us with a correct cDNA probe to be used for generation of larger EMMPRIN cDNAs.

After sequencing the small cDNA corresponding to peptide #51, a unique reverse complement primer derived from it was used in RT-PCR, together with a degenerate primer made from the amino terminal peptide #59, to obtain a longer cDNA. Sequencing of one of the isolated cDNAs (TALT5j; insert size, 0.6 kb) revealed the following characteristics: (*a*) as expected, the sequences at the 5′ and 3′ ends corresponded to the two primers, D and B′, which were based on portions of peptides #59 and #51, respectively; and (*b*) the complete sequences encoding peptides #26, #61, and #74 (Table 2) were present within the cDNA, demonstrating that the cDNA encodes an authentic EMMPRIN sequence.

An unexpected result from the above approach was that although the sequence of primer D was present at the 5′ end of the cDNA, the complete sequence corresponding to peptide #59, from which D is derived, was found to begin 72 nucleotide residues downstream from the primer sequence. Thus, it appears that primer D annealed with a region of the 5′ untranslated sequence of EMMPRIN and amplified a cDNA that begins in the 5′ untranslated region. A possible explanation of this event comes from comparison of the sequence of this cDNA with sequences recently obtained from an EMMPRIN cDNA derived from a human keratinocyte cDNA library.[7] The sequence at the 3′ end of primer D (Table 1) corresponds exactly with a sequence within the 5′ untranslated region, namely GAGGA, for the keratinocyte-derived as well as LX-1-derived EMMPRIN cDNAs. Therefore, this unexpected circumstance led to additional information about the 5′ untranslated end of our cDNA.

Thus, in summary, the 5′ cDNA (TALT5j) corresponds to nucleotide residues −14 to 536, defining +1 as A in the ATG start codon (Fig. 2). It begins with a portion of 5′ untranslated sequence, then contains a methionyl initiation codon at the start of a sequence encoding a region with the properties of a signal peptide (amino acid residues #1–21 in Fig. 2). This is followed by the sequence encoding peptide #59 (residues #22–36), corresponding to the NH_2-terminus of the mature protein. The cDNA continues through to the 3′ end primer, B′, encoding part of the amino acid sequence corresponding to peptide #51 (residues #173–178).

The rapid amplification of cDNA ends technique (24) was applied to obtain the 3′ cDNA, once more using a primer based on part of the authentic nucleotide sequence from the peptide #51 cDNA, together with a universal 3′ oligo dT_{17} adapter primer. Sequence analysis of the PCR products indicated that one of the inserts (TALT3g), 1.1 kb in size, begins at the 5′-terminus with the sequence corresponding to primer B, continues with the sequence of the COOH-terminus of peptide #51 (Table 1), and includes a perfect match with the amino acid sequence of peptide #38 (Table 2). The sequence of TALT3g corresponds to nucleotides 517–1592 in Fig. 2. This sequence also contains a stop codon at nucleotide positions #808–810 and a 3′ untranslated region containing a poly-adenylation signal and a poly(A) tail (Fig. 2).

Finally, the 5′ and 3′ cDNAs generated above were fused by the overlap extension method (25) to yield a single 1.6-kb cDNA. The full sequence of this cDNA, its deduced translation product, and the tryptic peptide sequences are shown in Fig. 2.

Northern Blot of mRNA for EMMPRIN. To ascertain the size of mRNA for EMMPRIN, total RNA derived from LX-1 cells was analyzed by Northern blotting using the 5′ cDNA (TALT5j) as a probe. A single band of ~1.7 kb was observed (Fig. 3). This mRNA size is close to the size of cDNA obtained after fusion of the TALT5j and TALT3g cDNAs, *i.e.*, 1.6 kb.

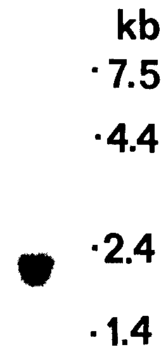

Fig. 3. Northern blot of LX-1 RNA probed with EMMPRIN cDNA. Ten μg of total RNA was loaded and probed with nick-translated, radiolabeled cDNA (TALT5j). Total radioactivity used was 1×10^6 cpm/ml, and the duration of film exposure was 18 h.

Analysis of EMMPRIN cDNA Sequences. The Northern blots indicate that the EMMPRIN composite cDNA corresponds to all but about 100 nucleotides of the mRNA. Since the clone contains a poly(A) tail, this means that the 100 bases are probably located at the 5′-end of the 5′ untranslated region, making the total untranslated region about 115 nucleotides in length.

The cDNA encodes a 269-amino acid residue polypeptide that contains a putative signal peptide of 21 amino acid residues, an extracellular domain of 185 amino acid residues, a putative transmembrane region (residues 206–229), and a carboxy-terminal cytoplasmic domain of 39 amino acid residues (230–269). The transmembrane region includes three leucines (residues 206, 213, and 220) and a phenylalanine (residue 227), occurring every seventh residue, a characteristic feature of the leucine zipper motif (Fig. 2). The extracellular region contains four cysteinyl residues spaced in a manner that gives rise to two distinct domains with the characteristics of proteins in the immunoglobulin superfamily. These residues in EMMPRIN are located at amino acid residue positions 41, 87, 126, and 185.

Expression of Recombinant EMMPRIN Protein and Deletion Constructs. Although the EMMPRIN cDNA encodes amino acid sequences identical to those of peptides directly isolated from immunoaffinity-purified EMMPRIN protein, we reconfirmed the identity of the EMMPRIN cDNA by showing that the recombinant protein is recognized by activity-blocking monoclonal antibody. To accomplish this, the recombinant protein was expressed in pBluescript and then assayed by Western blotting with EIIF4, a monoclonal antibody that blocks the activity of EMMPRIN protein from LX-1 cells (13, 14). As shown in Fig. 4*B*, *Lane 2*, EIIF4 reacts with the recombinant EMMPRIN protein. Two immunoreactive bands were obtained; these correspond in size to the two forms of EMMPRIN noted previously (14) and most likely arise by proteolysis.

The bacterial recombinant protein was also used to determine the approximate location of the epitope for E11F4, taking advantage of the lack of posttranslational modifications that would interfere with such studies on the native, immunoaffinity-purified protein. Modified EMMPRIN expression plasmids were made containing deletions in four locations. As seen in Fig. 4*A*, these deletions were: (*a*) deletion of the extracellular immunoglobulin domain I (ΔECI); (*b*) deletion of the extracellular immunoglobulin domain II (ΔECII); (*c*) deletion of the transmembrane domain (ΔTM); and (*d*) deletion of the cytoplasmic domain (ΔCYT). XL-1 blue cells were transformed with the deletion expression pBluescript plasmids, and the expressed proteins were analyzed by SDS-PAGE and Western blotting with the monoclonal antibody E11F4. As seen in Fig. 4*B*, all the plasmids produce protein that is immunoreactive, except for the plasmid lacking immunoglobulin domain I.

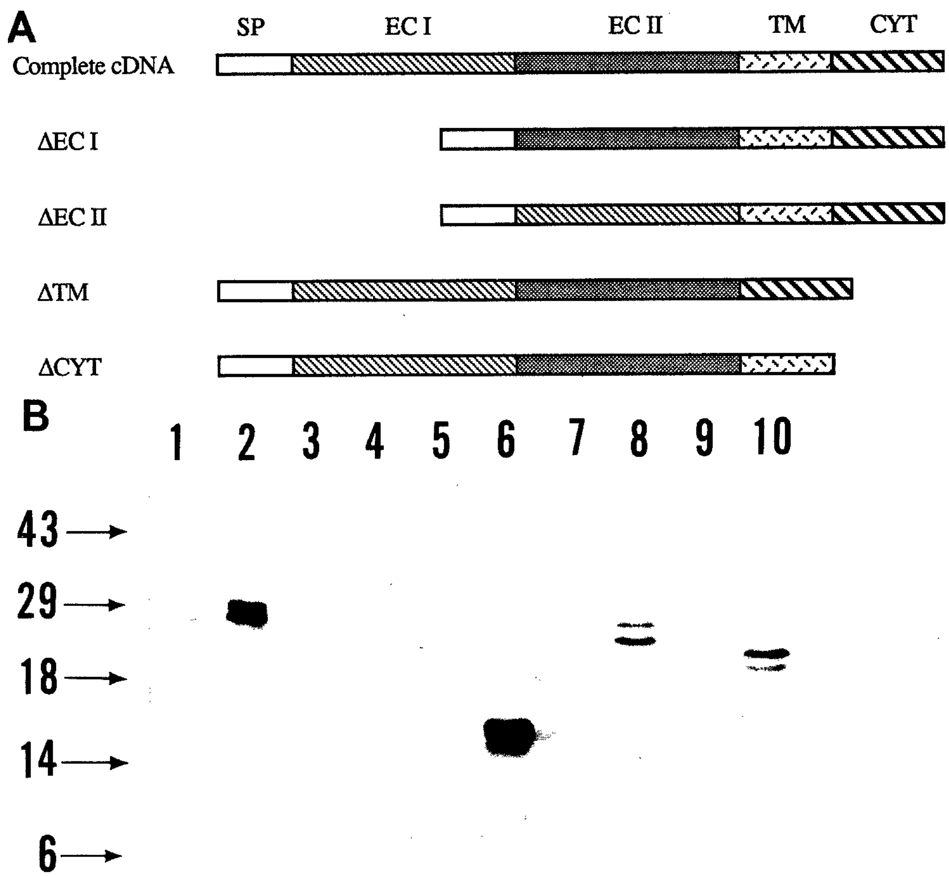

Fig. 4. Localization of EIIF4 epitope in recombinant EMMPRIN. *A*, strategy for deleting the four major domains of the EMMPRIN cDNA. ΔECI and ΔECII, deletion of one of two immunoglobulin-like domains within the extracellular region; ΔTM, deletion of transmembrane domain; ΔCYT, deletion of cytoplasmic domain; *SP*, signal peptide; *B*, Western blot. The transformed XL-1 blue cells were incubated in the absence (*Lanes 1, 3, 5, 7,* and *9*) or presence (*Lanes 2, 4, 6, 8,* and *10*) of 0.5 mM isopropyl-β-D-thiogalactopyranoside and processed for Western blotting. *Lanes 1* and *2*, recombinant EMMPRIN without deletion; *Lanes 3* and *4*, ΔECI; *Lanes 5* and *6*, ΔECII; *Lanes 7* and *8*, ΔTM; *Lanes 9* and *10*, ΔCYT. Molecular weight marker positions in kilodaltons are shown.

These results demonstrate that our cDNA encodes the protein which is reactive with our activity-blocking monoclonal antibody and that the antibody epitope exists in the extracellular immunoglobulin domain I. This, in turn, implies that the functional site of the metalloproteinase stimulatory activity of EMMPRIN is likely to be localized to sequences contained in the immunoglobulin domain I region.

DISCUSSION

We have isolated and fused two overlapping cDNA clones, using the polymerase chain reaction, which together encode the complete, 269-amino acid open reading frame for EMMPRIN. The identity of the clones was confirmed by comparison to several peptide sequences derived from immunoaffinity-purified EMMPRIN. Recognition of the translation product by the activity-blocking monoclonal antibody EIIF4 further confirms the identity of the cDNAs as the desired EMMPRIN clones. In addition, we have recently isolated an EMMPRIN cDNA from a human keratinocyte cDNA library. The open reading frame of the latter cDNA has an identical sequence to the cDNA obtained in the present study by PCR-based techniques, except for two nucleotide residues; however, the deduced amino acid sequences are identical for the two cDNAs.[7] Finally, we have recently demonstrated that recombinant EMMPRIN isolated from CHO cells transfected with EMMPRIN cDNA stimulates metalloproteinase production in fibroblasts.[9]

The composite cDNA obtained in the present study has a small 5′ untranslated region, followed by an initiation codon and sequences that have the properties of a signal peptide sequence, when using the rules of von Heijne (27). The subsequent codons agree perfectly with our amino terminal peptide sequence for the mature protein, demonstrating that the signal peptide sequence is genuine. The 248 codons after the signal sequence encode a 185-amino acid extracellular domain consisting of two regions characteristic of the immunoglobulin superfamily, followed by 24-amino acid residues comprising the transmembrane domain and a 39-amino acid cytoplasmic domain. The 248-amino acid residues of the mature protein correspond to an approximate molecular weight of 27,000. However, the purified protein has a larger molecular weight of ~58,000 (13). This difference is mainly due to glycosylation of the protein[7] (20).

We have shown previously that EMMPRIN is present at the surface of tumor cells (13) and has the properties of a membrane-intercalated protein (12). On the basis of these findings, we proposed previously that EMMPRIN is attached to the plasma membrane via a transmembrane domain and interacts with a receptor on fibroblasts via an extracellular domain (1). The presence in the cDNA of sequences typical of a signal peptide and a transmembrane region is consistent with EMMPRIN being an integral plasma membrane protein.

After the termination codon, the cDNA contains a 3′ untranslated region ending in a poly(A) tail. Northern blot analysis indicates that the mRNA for EMMPRIN is ~1.7 kb in size, which is approximately the same as that of the fused EMMPRIN cDNA. It is evident, however, that a portion of the 5′ untranslated region is lacking from this cDNA.

The EMMPRIN cDNA sequences were used in computer searches of the EMBL and GenBank data bases to detect homology with other known proteins. These searches revealed that the EMMPRIN cDNA is identical to two other human cDNAs, encoding proteins of un-

[9] H. Guo, M. Gordon, B. P. Toole, and C. Biswas, Recombinant human tumor cell EMMPRIN stimulates fibroblast metalloproteinase production, manuscript in preparation.

known function, basigin (19) and the M6 antigen (20). Therefore, our studies, which have been performed from a functional standpoint over the course of many years (4, 6, 12–16), have elucidated at least one biological function of these molecules. We are changing our former designation of the molecule from TCSF to EMMPRIN and would suggest that all the above proteins now be designated EMMPRIN, because the acronym more accurately implies at least one definitive function of the glycoprotein, *i.e.*, stimulation of MMP synthesis via cell-cell interaction.

The fact that EMMPRIN is a member of the immunoglobulin superfamily is also compatible with the idea that, in similar fashion to the N-CAM, I-CAM, and other related subgroups of the immunoglobulin superfamily (28), it acts via cell-cell interactions (1). We are currently attempting to identify the molecule on the surface of fibroblasts that interacts with tumor cell-derived EMMPRIN, causing increased fibroblast MMP production. Our recent finding that EMMPRIN is expressed in keratinocytes and localized in the basal layers of the epidermis[7] suggests the possibility that EMMPRIN may have a natural function in embryonic development or wound healing by causing dermal fibroblasts to increase their MMP production, thus facilitating tissue remodeling (18). The antibody to M6 antigen localizes EMMPRIN to granulocytes in patients with rheumatoid arthritis (20), possibly indicating a role for EMMPRIN in stromal MMP production and the consequent matrix degradation that occurs in the arthritic joint. Thus, we propose that EMMPRIN and related molecules are important mediators of matrix remodeling in normal and pathological tissues.

With respect to tumorigenesis, it has become clear that: (*a*) MMPs are crucial to the process of tumor cell invasion through basement membranes and interstitial matrices (1–3); and (*b*) in the case of interstitial collagenase, stromelysin, and M_r 72,000 gelatinase (type IV collagenase), the MMPs involved are produced mainly by peritumoral fibroblasts rather than by the tumor cells themselves (8–11). Since tumor cell-derived EMMPRIN causes a significant increase in the levels of these three enzymes in human fibroblasts (6, 13–16) and since EMMPRIN is associated with the surface of many types of tumor cells *in vivo* and *in vitro* (13, 17),[10] it is very likely that EMMPRIN is a central factor in the stimulation of MMPs required for tumor invasion and metastasis.

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Communication

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Stimulation of Matrix Metalloproteinase Production by Recombinant Extracellular Matrix Metalloproteinase Inducer from Transfected Chinese Hamster Ovary Cells*

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Many of the tumor-associated matrix metalloproteinases that are implicated in metastasis are produced by stromal fibroblasts within or surrounding the tumor in response to stimulation by factors produced by tumor cells. In this study we transfected Chinese hamster ovary cells with putative cDNA for human extracellular matrix metalloproteinase inducer (EMMPRIN), a transmembrane glycoprotein that is attached to the surface of many types of malignant human tumor cells and that has previously been implicated in stimulation of matrix metalloproteinase production in fibroblasts. We show that these transfected cells synthesize EMMPRIN that is extensively post-translationally processed; this recombinant EMMPRIN stimulates human fibroblast production of interstitial collagenase, stromelysin-1, and gelatinase A (72-kDa type IV collagenase). We propose that EMMPRIN regulates matrix metalloproteinase production during tumor invasion and other processes involving tissue remodeling.

Successful tumor metastasis requires many steps, one of which is local proteolytic destruction of extracellular matrix at sites of tumor invasion. A major class of proteinases associated with tumor invasion is the matrix metalloproteinases (MMPs)[1] (1, 2). Although it was initially thought that these enzymes were mainly produced by malignant tumor cells themselves, it is now clear that interstitial collagenase (MMP-1), gelatinase A (MMP-2, a 72-kDa type IV collagenase), and stromelysin-1 (MMP-3) are produced *in vivo* by stromal fibroblasts associated with several types of tumors (2–7). MMP-2 synthesized and secreted by these fibroblasts has been shown to adhere to the surface of tumor cells, facilitating tissue invasion (8–10). Because quiescent fibroblasts generally produce relatively low amounts of MMPs (11, 12), tumor-associated fibroblasts must be influenced in some way to give rise to the elevated levels of MMPs usually present in malignant tumors. One possibility that we have investigated is that tumor cells interact with fibroblasts via soluble or cell-bound factors, stimulating fibroblast MMP production (11–15). Our studies have led to characterization of a tumor cell surface protein, extracellular matrix metalloproteinase inducer (EMMPRIN; previously termed tumor cell-derived collagenase stimulatory factor or TCSF), that stimulates fibroblast production of MMP-1, MMP-2, and MMP-3 (12–14). We recently obtained cDNAs for human EMMPRIN and verified their identity by recognition of recombinant EMMPRIN by activity-blocking monoclonal antibody and by sequence identity with amino acid sequences of peptides isolated from EMMPRIN (15). However, recombinant EMMPRIN produced by bacteria is much smaller than native EMMPRIN isolated from tumor cells because it is not post-translationally processed. This form of recombinant EMMPRIN is inactive, thus leaving some doubt regarding the identity of the cDNAs. In this study, we use the cDNAs to transfect CHO cells and show that EMMPRIN produced by these transfected cells is post-translationally processed and stimulates fibroblasts to produce elevated levels of MMP-1, MMP-2, and MMP-3.

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† This article is dedicated to the memory of our friend and colleague, Chitra, in whose laboratory much of this work was done but who died in August, 1993.

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[1] The abbreviations used are: MMP, matrix metalloproteinase; EMMPRIN, extracellular matrix metalloproteinase inducer; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate ester; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

EXPERIMENTAL PROCEDURES

Stable Transfection of CHO Cells with EMMPRIN cDNA—EMMPRIN cDNA (15) was subcloned into an expression vector, pcDNA/Neo (Invitrogen, San Diego, CA), and purified by CsCl gradient centrifugation and phenol/chloroform extraction. CHO cells (American Type Culture Collection, Bethesda, MD) were seeded at 10^6 cells/100-mm tissue culture dish and incubated overnight, at which stage they were 50–70% confluent. The cells then were transfected in 5 ml of serum-free Ham's F-12 medium containing lipofectamine-DNA complex (10 μl of lipofectamine (Life Technologies, Inc.) mixed with 10 μg of DNA with or without the EMMPRIN insert). After 6 h of incubation at 37 °C, 5 ml of medium containing 20% fetal bovine serum was added to the transfection mixture, which was then cultured at 37 °C for a further 72 h. The cells then were treated with trypsin-EDTA (Life Technologies, Inc.) and subcultured in medium containing 400 mg/liter of Geneticin (Life Technologies, Inc.) for 2–3 weeks. Successful transfection was assessed by immunocytochemistry using monoclonal antibody E11F4 raised against EMMPRIN, as described previously (13).

Purification of EMMPRIN—EMMPRIN was purified from detergent extracts of cell membranes from LX-1 cells or stably transfected CHO cells by immunoaffinity chromatography using monoclonal antibody E11F4 against EMMPRIN as described previously (13). Briefly, the cell membranes were extracted with 10 mM Tris-HCl buffer (pH 8.2), containing 0.5% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. The supernatant of the extract was then applied to a 5-ml anti-EMMPRIN affinity column and recirculated through the column for 12 h at 4 °C. The column was washed with buffer several times, and EMMPRIN was then eluted from the column with 50 mM diethylamine, 30 mM octylglucoside (pH 11.5). The eluted protein was neutralized with 0.5 M NaH_2PO_4, dialyzed against 0.1 M acetic acid, concentrated, and dissolved in 0.1 M acetic acid.

Assays for EMMPRIN Activity—Human fibroblasts (isolated from

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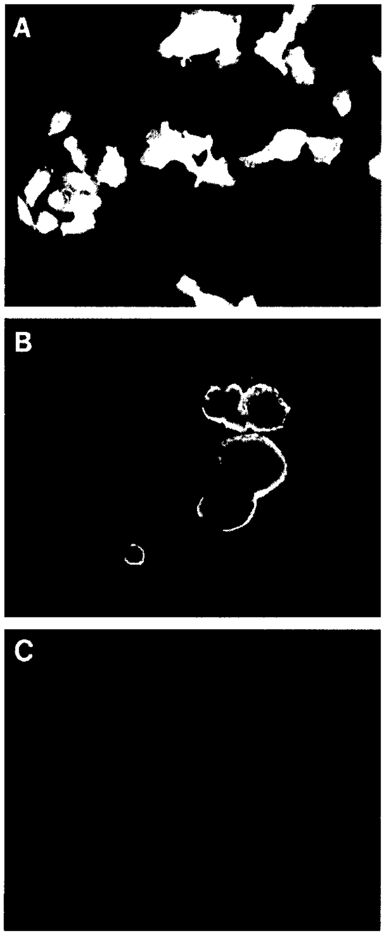

FIG. 1. **Immunofluorescent staining of CHO cells transfected with EMMPRIN cDNA.** Staining was carried out using monoclonal antibody, E11F4, against EMMPRIN as described previously (13). *A*, CHO cells transfected with EMMPRIN cDNA, fixed under normal culture conditions. *B*, similar cells to those in *A*, but fixed 4 h after plating. *C*, CHO cells mock-transfected with vector. Untransfected CHO cells also show no reactivity with E11F4 (not shown).

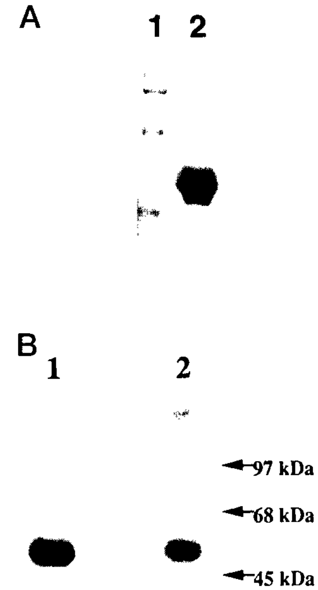

FIG. 2. **SDS-PAGE and Western blotting of purified recombinant EMMPRIN.** *A*, silver-stained SDS-PAGE gel of EMMPRIN purified from CHO cells transfected with EMMPRIN cDNA. EMMPRIN was purified from cell membranes as described under "Experimental Procedures," dissolved in SDS sample buffer containing 0.1 M dithiothreitol, heated at 95 °C for 10 min, and subjected to 10% SDS-PAGE; the gel was deliberately overloaded to reveal potential contaminants. *Lane 1*, molecular mass standards (45, 66, 97, and 116 kDa); *lane 2*, purified recombinant EMMPRIN. *B*, Western blot of recombinant EMMPRIN purified from CHO cells transfected with EMMPRIN cDNA (*lane 1*) and of native EMMPRIN purified from LX-1 cells (*lane 2*). A 10% SDS-PAGE gel was electroblotted to a nitrocellulose membrane followed by blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20. The blot was incubated with E11F4 hybridoma supernatant (13) for 1 h at room temperature and then with horseradish peroxidase-conjugated anti-mouse IgG. The EMMPRIN protein bands were detected with ECL Western blotting detection reagents (Amersham Corp.). In both cases the anti-EMMPRIN antibody recognized a protein with a molecular mass of ~58 kDa. Some immunoreactive, aggregated protein was also present in LX-1 cells, as previously noted (14).

human skin in our laboratory) were cultured for 24 h in 24-well plates in 1 ml of DMEM medium supplemented with 10% fetal bovine serum, after which the medium was replaced with 0.5 ml of DMEM containing 2% fetal bovine serum in the presence or the absence of EMMPRIN or TPA, and the cultures were further incubated at 37 °C for 3 days. Media from these cultures were used for zymographic assay of MMP-3 (16) and ELISA of MMP-1, MMP-2, and MMP-3 (12, 17).

RESULTS

In initial attempts to demonstrate recombinant EMMPRIN activity we tested purified, pGEX bacterial expression protein. However, EMMPRIN produced in the pGEX system had a molecular mass of only ~29 kDa (equivalent to that expected from the cDNA open reading frame (15)), compared with native EMMPRIN from tumor cells, which is ~58 kDa (12–14). This bacterially produced recombinant EMMPRIN protein was inactive in stimulating MMP production by human fibroblasts. Next, COS and CHO cells were transfected with EMMPRIN cDNA under a variety of conditions, but in most cases the EMMPRIN produced was either of similar molecular mass to bacterial recombinant protein, *i.e.* ~29 kDa, or was partially post-translationally processed with molecular masses ranging from 30–45 kDa. EMMPRIN isolated from these cells was also inactive.

However we found that after stable transfection (see "Experimental Procedures"), CHO cells could be selected that synthesize high levels of EMMPRIN of similar molecular mass to that of native EMMPRIN isolated directly from LX-1 human carcinoma cells. Fig. 1*A* shows detection of this recombinant EMMPRIN in the transfected CHO cells by immunocytochemistry using monoclonal antibody raised against native EMMPRIN from LX-1 cells (13). Because the transfected cells are very flat, it is difficult to discern the precise cellular distribution of EMMPRIN. However, if the cells are fixed shortly after plating (~4 h), *i.e.* before they have flattened, it is clear that EMMPRIN is located at the surface of the transfected cells (Fig. 1*B*). Untransfected cells or cells that are mock-transfected with vector only show no reactivity with the antibody (Fig. 1*C*).

Fig. 2*A* shows a silver-stained SDS-PAGE gel of recombinant EMMPRIN purified by immunoaffinity chromatography from membrane extracts of the stably transfected CHO cells; this gel was deliberately overloaded to reveal potential contaminants in the preparation. A single broad band at ~58 kDa was detected, as previously obtained for tumor cell-derived EMMPRIN (13, 14). Direct comparison of purified recombinant and LX-1 carcinoma cell-derived EMMPRIN by Western blotting showed that they were identical in size (Fig. 2*B*), indicating that the recombinant EMMPRIN was fully or almost fully post-translationally processed. Untransfected CHO cells or cells transfected with vector only did not produce any EMMPRIN detectable by immunoaffinity chromatography and Western blotting (not shown).

We then tested purified recombinant EMMPRIN from transfected CHO cells for its ability to stimulate MMP production by human fibroblasts in culture. We first measured the effect of recombinant EMMPRIN on MMP-3 production by zymography,

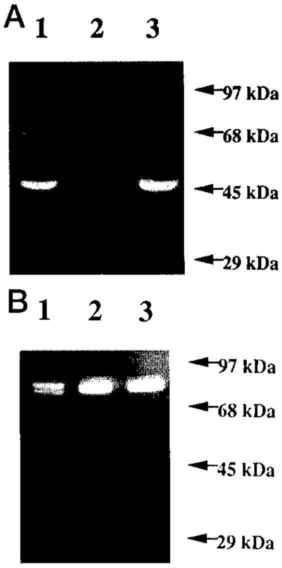

FIG. 3. **Stimulation of stromelysin-1 (MMP-3) production in human fibroblasts incubated with recombinant EMMPRIN isolated from transfected CHO cells.** Media (15 μl each) from fibroblasts cultured in the presence or the absence of EMMPRIN or TPA were subjected to electrophoresis without reduction in 10% SDS-polyacrylamide impregnated with 0.5 mg/ml β-casein (*A*) or 0.5 mg/ml carboxymethylated transferrin (*B*). After electrophoresis, the SDS was eluted from the gels in 2.5% Triton X-100 for 30 min at 25 °C, and the gels were then incubated 20 h in substrate buffer containing 50 mM Tris-HCl and 5 mM $CaCl_2$. The gels were stained with Coomassie Blue, and the presence of proteolytic enzymes was identified by the appearance of clear zones where the casein or transferrin substrate had been digested. *Lane 1*, fibroblasts incubated with recombinant EMMPRIN (100 μg/ml); *lane 2*, fibroblasts incubated without added protein; *lane 3*, fibroblasts incubated with TPA (0.1 μg/ml). The clear band at ~45 kDa present in *lanes 1* and *3*, but in much lower amount in *lane 2*, represents MMP-3. The other clear zones are due to other proteolytic enzymes constitutive to the fibroblasts.

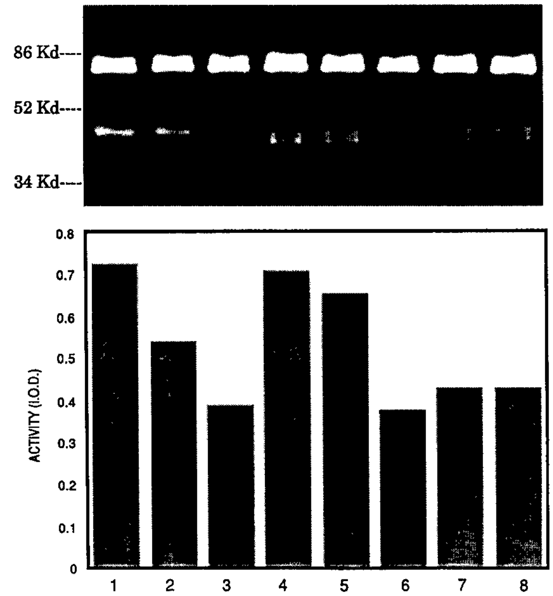

FIG. 4. **Effect of antibody against native EMMPRIN on stimulation of MMP-3 production by recombinant EMMPRIN.** MMP-3 was detected in the same fashion as described in the legend to Fig. 3*B* using 0.5 mg/ml of carboxymethylated transferrin as substrate. The *top panel* shows the zymogram; the *bottom panel* shows quantitation of the ~45-kDa band (or doublet) in each lane by densitometry using a Bio Image Whole Band Analysis package. *Lane 1*, plus 100 ng/ml TPA; *lane 2*, plus 50 ng/ml TPA; *lane 3*, no addition; *lanes 4* and *5*, plus 100 μg/ml recombinant EMMPRIN; *lane 6*, plus supernatant derived after protein A treatment of medium containing 100 μg/ml recombinant EMMPRIN and EIIF4 monoclonal antibody against EMMPRIN, as described previously (13); *lane 7*, plus 100 μg/ml recombinant EMMPRIN and antibody EIIF4; *lane 8*, no addition.

using two separate substrates, casein (Fig. 3*A*) and carboxymethylated transferrin (Fig. 3*B*). A clear-cut increase in active MMP-3 was observed in fibroblasts treated with the recombinant EMMPRIN (Fig. 3, *A* and *B*, *lane 1 versus 2*). The amount of MMP-3 was similar to that induced by TPA treatment (Fig. 3, *A* and *B*, *lane 3*).

To ensure that stimulation of MMP-3 production was not due to minor contaminants in the recombinant EMMPRIN preparation, we tested the effect of blocking antibody raised against native EMMPRIN (13) on the stimulation by recombinant EMMPRIN, using two different approaches. In the first approach, antibody was included in the culture medium together with EMMPRIN throughout the 3-day incubation period (Fig. 4, *lane 7*). In the second approach, the antibody was mixed with EMMPRIN and then removed by binding to protein A; the supernatant from this reaction, depleted of antigen, was then added to the culture for the 3-day incubation (Fig. 4, *lane 6*). As shown in Fig. 4, the stimulation of stromelysin production in cells treated with EMMPRIN (*lanes 4* and *5 versus lanes 3* and *8*) was completely reversed by either of the two different treatments with antibody to EMMPRIN (*lanes 6* and *7*).

Finally, we measured the effect of recombinant EMMPRIN on MMP-1, MMP-2, and MMP-3 production by ELISA. In two separate experiments, treatment of fibroblasts with the EMMPRIN gave rise to significant increments in production of MMP-1 (~6- and ~11-fold), MMP-2 (~1.5- and ~16-fold), and MMP-3 (~2- and ~4-fold) (Table I). In most cases the degree of stimulation of MMP by recombinant EMMPRIN was similar to that caused by TPA (Table I).

DISCUSSION

The results presented here constitute final proof that the cDNAs that we have obtained genuinely encode EMMPRIN, as defined by its MMP stimulatory activity. The degree of stimulation of MMP-1, MMP-2, and MMP-3 by recombinant EMMPRIN obtained herein is similar to that obtained previously with native EMMPRIN purified from LX-1 carcinoma cells (12). It should be pointed out, however, that the degree of stimulation by either native or recombinant EMMPRIN varies in different fibroblast preparations. First, some fibroblast preparations are not significantly responsive to EMMPRIN stimulation, whereas others are very responsive (*e.g.* see Ref. 12). Second, among populations of fibroblasts that are responsive to EMMPRIN, an important variable is the extent to which a batch of fibroblasts already produces a given MMP without addition of stimulatory agents. This can be readily appreciated for MMP-3 production in Fig. 3*B*, *lane 2*, versus Fig. 4, *lanes 3* and *8*, and for MMP-2 production in Table I, experiment 1 *versus* experiment 2. Our overall experience has been that EMMPRIN stimulation of MMP-1 and MMP-3 production is usually in the range of 3–10-fold. Stimulation of MMP-2, however, is even more variable (*e.g.* see Table I, experiment 1 *versus* 2) but is usually rather modest, *e.g.* 1.5–2-fold. One reason for this variability is clearly the significant amounts of MMP-2 that many fibroblast preparations produce without treatment with exogenous agents, *e.g.* experiment 1 in Table I. Also, however, the mechanism of stimulation of MMP-2 may be more complex than for MMP-1 and MMP-3. As well as stimulating overall MMP-2 production, EMMPRIN apparently enhances activation of MMP-2, and this sometimes leads to underestimation of MMP-2 stimulation in ELISA assays (12).

The experiments described here indicate that EMMPRIN activity is dependent on post-translational processing. How-

TABLE I
Stimulation of MMP production by recombinant EMMPRIN

Recombinant EMMPRIN was purified from membranes of transfected CHO cells and, in two separate experiments, added at 100 μg/ml to cultures of human fibroblasts. Cultures were also incubated with TPA (0.1 μg/ml) or with no added reagent. After incubation, aliquots of culture medium were used for ELISA of MMP-1, MMP-2, and MMP-3. Amounts of MMP are expressed as μg/ml ± S.E.

Agent added	MMP-1	MMP-2	MMP-3
Experiment 1			
None	0.03 ± 0.00	1.40 ± 0.01	0.21 ± 0.01
rEMMPRIN	0.33 ± 0.02[a]	2.12 ± 0.13[a]	0.93 ± 0.13[a]
TPA	0.32 ± 0.02[a]	2.33 ± 0.29[a]	0.42 ± 0.02[a]
Experiment 2			
None	0.03 ± 0.00	0.13 ± 0.01	0.35 ± 0.04
rEMMPRIN	0.17 ± 0.02[a]	2.10 ± 0.37[a]	0.63 ± 0.03[a]
TPA	0.25 ± 0.02[a]	0.46 ± 0.06[a]	0.56 ± 0.07[a]

[a] Significantly greater than control (none added), $p < 0.05$.

ever, it is not yet clear whether processing is required to attain the appropriate conformation for activity or whether specific side groups, *e.g.* carbohydrate or phosphate, are involved in EMMPRIN receptor recognition.

We have shown by immunocytochemistry that EMMPRIN is present on the surface of tumor cells but not fibroblasts and several other normal adult cell types (13, 18, 19). Prior studies have also shown that tumor cells shed EMMPRIN (13) and that EMMPRIN appears in the urine of bladder carcinoma patients (18, 20). Thus tumor cell EMMPRIN, in soluble or membrane-bound form, is likely to be responsible for at least part of the stimulation of fibroblast MMP production observed *in vivo* in association with a variety of malignant tumors (3–7). In preliminary experiments, we have found that production of the tissue inhibitor of matrix metalloproteinases, TIMP-1, is not stimulated by EMMPRIN; if this proves to be true *in vivo*, an imbalance of active *versus* inactive MMP production may result from EMMPRIN action on stromal fibroblasts.

The amino acid sequence of EMMPRIN is identical to that of human M6 antigen (21) and basigin (22), for which no function had previously been ascribed until EMMPRIN was cloned (15). Several proteins with high levels of homology to EMMPRIN, *i.e.* neurothelin, HT7, OX47, and gp42, have also been characterized in other species (21–23), but again their function has not been determined. These proteins may be species homologues of one another or closely related members of a protein family; it remains to be seen whether all of these proteins have EMMPRIN activity.

The ability of EMMPRIN to stimulate MMP production has led us to propose that it may be involved in a wide range of physiological and pathological processes where tissue remodeling takes place (24). For example, its presence in the epidermis (25) and in several embryonic epithelia[2] suggests that EMMPRIN may participate in epithelial-mesenchymal interactions leading to changes in tissue architecture during development and wound healing. Also, EMMPRIN on the surface of activated lymphocytes and monocytes (21) may contribute to elevated MMP levels found in arthritis. However, association of EMMPRIN-like material with endothelium during formation of the blood-brain barrier and with highly organized epithelia such as retina and kidney tubules (23) suggests that it may have additional functions involving cell-cell interactions.

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ARTICLE

Tumor Collagenase Stimulatory Factor (TCSF) Expression and Localization in Human Lung and Breast Cancers

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SUMMARY Tumor cell-derived collagenase stimulatory factor (TCSF) stimulates in vitro the biosynthesis of various matrix metalloproteinases involved in tumor invasion, such as interstitial collagenase, gelatinase A, and stromelysin 1. The expression of TCSF mRNAs was studied in vivo, using in situ hybridization and Northern blotting analysis, in seven normal tissues and in 22 squamous cell carcinomas of the lung, and in seven benign proliferations and in 22 ductal carcinomas of the mammary gland. By in situ hybridization, TCSF mRNAs were detected in 40 of 44 carcinomas, in pre-invasive and invasive cancer cells of both lung and breast cancers. TCSF mRNAs and gelatinase A mRNAs were both visualized in the same areas in serial sections in breast cancers, and were expressed by different cells, tumor cells, and fibroblasts. The histological results were confirmed by Northern blot analysis, which showed a higher expression of TCSF mRNAs in cancers than in benign and normal tissues. These observations support the hypothesis that TCSF is an important factor in lung and breast tumor progression. (J Histochem Cytochem 45:703–709, 1997)

KEY WORDS
TCSF
metalloproteinases
tumor invasion

TUMOR INVASION is a multistep process that involves the degradation of basement membrane and interstitial matrix components by proteolytic enzymes. Many data actually support an important role for the matrix metalloproteinases (MMPs) in this proteolytic event. High levels of MMPs have been described in many cancer cell lines that display high invasive capacity (Gilles et al. 1994; Monsky et al. 1994; Taniguchi et al. 1992,1994; Bernhard et al. 1990; Bonfil et al. 1989). Such an observation has been recently extended to a newly discovered member of the MMPs, membrane type matrix metalloproteinase 1 (MT-MMP-1), which has also been shown to be correlated with in vitro invasiveness (Sato et al. 1994; Okada et al. 1995; Gilles et al. 1996). In vivo, MMPs have also been associated with the metastatic progression of many human cancers (Davies et al. 1993; Clavel et al. 1992; Levy et al. 1991; Monteagudo et al. 1990). However, recent in vivo data obtained by in situ hybridization (ISH) have shown that interstitial collagenase, gelatinase A, stromelysins, and MT-MMP-1 are mostly synthesized by fibroblasts localized near tumor cell clusters (Okada et al. 1995; Pyke et al. 1993; Poulsom et al. 1992,1993; Polette et al. 1991,1993,1996; Basset et al. 1990).

The specific detection of MMPs in peritumoral fibroblasts has led to the hypothesis that tumor cells might induce the synthesis of these enzymes implicated in cancer dissemination. In agreement with such an idea, several investigators have demonstrated cooperation between tumor cells and fibroblasts in vitro in the regulation of several MMPs, such as interstitial collagenase (Noël et al. 1993; Hernandez et al. 1985; Biswas 1984,1985; Bauer et al. 1979) and gelatinase A (Ito et al. 1995; Noël et al. 1994). Furthermore, a tumor cell-derived collagenase stimulatory factor (TCSF) also present in tumor cell-conditioned media, was isolated and purified from the plasma membranes of a human lung carcinoma cell line (Ellis et al. 1989). This factor is a glycoprotein of 58 kD and was recently

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identified as a member of the immunoglobulin superfamily (Biswas et al. 1995). In addition to enhancing interstitial collagenase synthesis (Nabeshima et al. 1991), purified TCSF stimulates gelatinase A and stromelysin 1 expression by fibroblasts (Kataoka et al. 1993). Immunohistochemical studies employing a monoclonal antibody directed against TCSF have shown that TCSF is localized to the outer surface of cultured lung cancer cell lines (Ellis et al. 1989). The same distribution was seen in tumors of urinary bladder, in which TCSF was detected at the periphery of cancer cells but not in surrounding stromal cells (Muraoka et al. 1993). Furthermore, TCSF was also localized in tumor cells by immunohistochemistry in invasive and in situ ductal breast cancers (Zucker and Biswas 1994).

On the basis of limited information concerning TCSF localization in cancer tissue, the role of TCSF in tumor progression remains unclear. In the present study, to clarify the cell origin of TCSF and to study its role in cancer invasion, we performed in situ hybridization and Northern blot analysis on human lung and breast carcinomas as well as on normal tissues.

Materials and Methods

Source of Tissue

The tissue was obtained from 22 lungs resected for squamous cell carcinomas of Stages I (10 cases), II (eight cases), and III (four cases) according to the TMN classification, from seven normal lung samples, from 22 ductal breast cancers of Grade 1 (four cases), Grade 2 (14 cases), and Grade 3 (four cases) according to the Scarf and Bloom classification, and from seven benign breast proliferations (two fibrocystic disease and five fibroadenoma).

Tissue Preparation

Part of the samples were frozen in liquid nitrogen for Northern blot analysis and the remainder were fixed in formalin and embedded in paraffin for in situ hybridization.

In Situ Hybridization Localization

Tissue sections (5 μm) were deparaffinized, rehydrated, and treated with 0.2 M HCl for 20 min at room temperature, followed by 15 min in 1 μg/ml proteinase K (Sigma Chemical; St Louis, MO) in Tris–EDTA–NaCl, 37C, to remove basic proteins. The sections were washed in 2 × SSC (sodium saline citrate), acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, and hybridized overnight with ^{35}S-labeled (50C) anti-sense RNA transcripts. TCSF cDNA (1700 BP) and gelatinase A (1500 BP) (a gift from G. Murphy; Cambridge, UK) were subcloned into pBluescript II SK+/− plasmid and pSP64, respectively, and used to prepare ^{35}S-labeled RNA probes. Hybridizations were followed by RNAse treatment (20 μg/ml, 1 h, 37C) to remove unhybridized probe and two stringent washes (50% formamide–2 × SSC, 2 hr at 60C) before autoradiography using D 19 emulsion (Kodak; Rochester, NY). Slides were exposed for 15 days before development. The controls were performed under the same conditions, using ^{35}S-labeled sense RNA probes. All slides were counterstained with HPS (hematoxylin–phloxin–safran), mounted, and examined under a Zeiss Axiophot microscope.

In Situ Hybridization Quantitation by Image Cytometry

Quantitation of the number of hybridization grains/μm^2 was performed with the help of an automated image analyzer, the DISCOVERY system (Becton–Dickinson; Mountain View, CA). After thresholding, the number of grains are counted automatically on at least six fields at high magnification (× 500). At this magnification, one field measures 12,688 μm^2. We performed these measurements on six different samples (three lung and three breast carcinomas) in which we found normal, in situ, and invasive areas on the same tissue section. Statistical analyses of TCSF mRNA expression levels were compared using the non-parametric Mann–Whitney *U*-test. Data were expressed as mean of dots/μm^2 ± SEM. p values equal to or less than 0.05 were considered significant.

Northern Blot Analysis

Extraction of total RNA from tissues was performed by RNAzol treatment (Biogenesis; Bournemouth, UK). Ten μg of each RNA was analyzed by electrophoresis in 1% agarose gels containing 10% formaldehyde and transferred onto nylon membranes (Hybond-N; Amersham, Poole, UK). The membrane was hybridized with the cDNA probe encoding TCSF (1700 BP) labeled with ^{32}P using random priming synthesis (5 × 108 cpm/μg) (Dupont de Nemours; Bruxelles, Belgium). The filters were exposed for 1 day. Membranes were rehybridized to a ubiquitous 36B4 gene probe, which served as a control. Signal intensities were recorded using a CD 60 Desaga (Heidelberg, Germany) laser-scanning densitometer and TCSF levels (in arbitrary units) were standardized with their corresponding 36B4 levels to obtain values independent of RNA quantities deposed onto gels. Statistical analyses of TCSF expression levels were compared using the non-parametric Mann–Whitney *U*-test. Data were expressed as mean ± SEM. Differences or similarities between two populations were considered significant when confidence intervals were <95% (p<0.05).

Results

Lung Lesions

By Northern blotting, TCSF transcripts were detected in 18 of 22 carcinomas. Quantitative analysis showed significantly higher (p<0.05) TCSF mRNA expression in lung carcinomas than in peritumoral lung tissues (Figures 1 and 2A). However, no significant differences between the TCSF mRNA levels were found in accordance with the TNM stage (Figure 2A).

With in situ hybridization, pre-invasive and invasive cancer cells were labeled in 18 of 22 tumors examined (the same positive samples as those found by

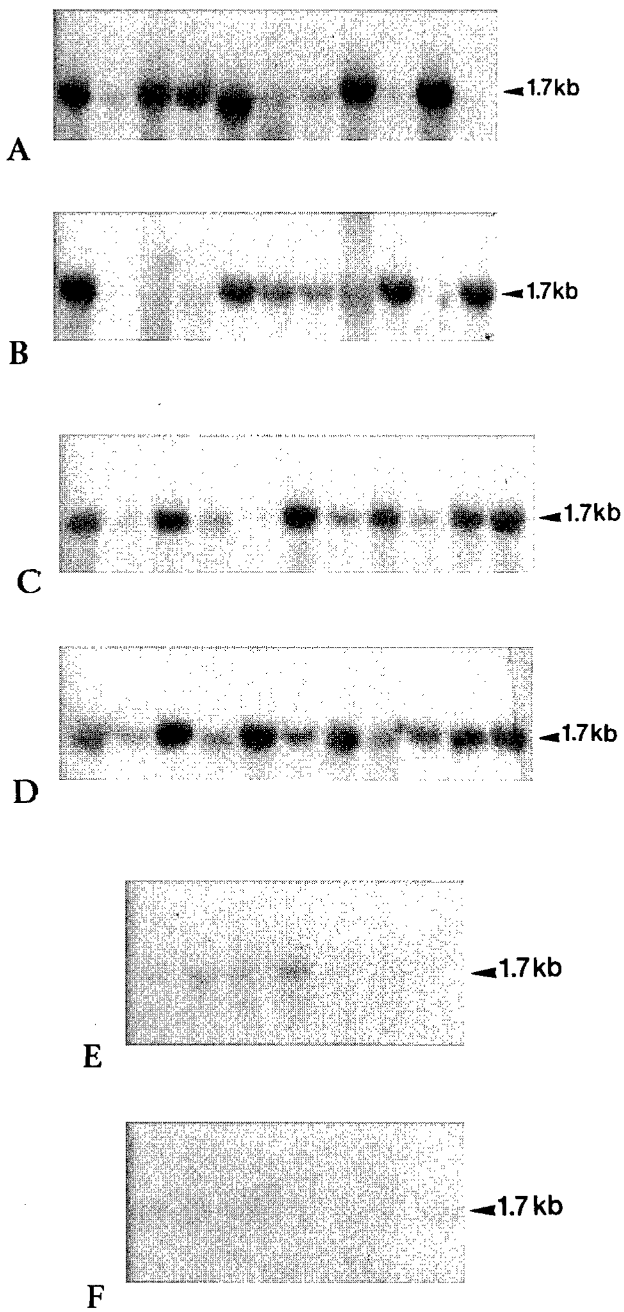

Figure 1 Northern blotting analysis of total RNA extracted from 22 lung carcinomas (**A,B**), 22 breast carcinomas (**C,D**), seven peritumoral normal lung tissue samples (**E**), and seven benign breast proliferations (**F**). A TCSF transcript of 1.7 KB is detected in tumor samples (40/44), whereas it is very weak or undetectable in normal tissue or benign proliferations.

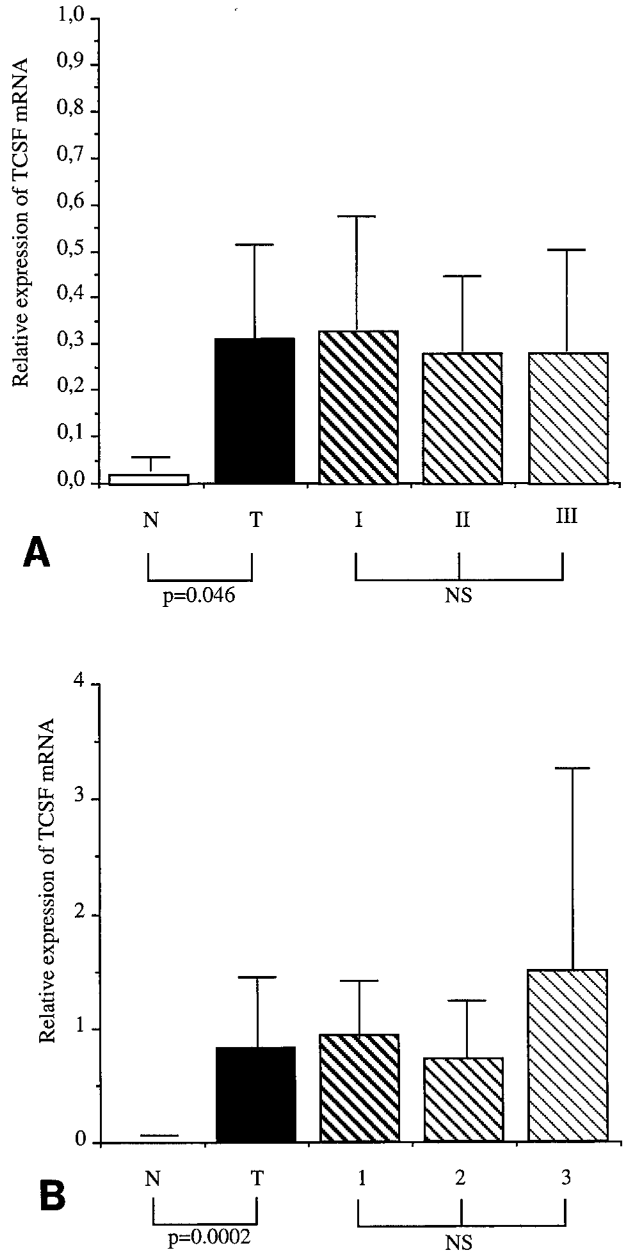

Figure 2 Comparison of TCSF mRNA levels (arbitrary units) according to tissue pathology and to the TNM stage and Scarf and Bloom grading in lung (**A**) and breast (**B**) samples. (**A**) Lung tumor samples (T) expressed significant higher TCSF mRNA levels than non-tumor samples (N). However, no significant differences were found according to the TNM stage of lung carcinomas. (**B**) Breast tumor samples (T) expressed high TCSF mRNA levels whereas no signal was detected in benign breast tissues (N). Statistical analysis did not find any significant differences according to the Scarf and Bloom grade of breast carcinomas.

Northern blot analysis). Stromal cells surrounding labeled invasive cancer cells, were always negative (Figure 3A). Normal (Figure 3C) or squamous metaplastic epithelium and bronchial glands did not express any TCSF transcripts. Moreover, in the normal or emphysematous adjacent lung, pulmonary alveolar macrophages identified by the CD68 monoclonal antibody (Dako; Carpinteria, CA) on serial sections (not shown) were particularly rich in TCSF mRNAs (Figure 3D). In the three cases analyzed by image cytometry, TCSF mRNAs were significantly expressed in tu-

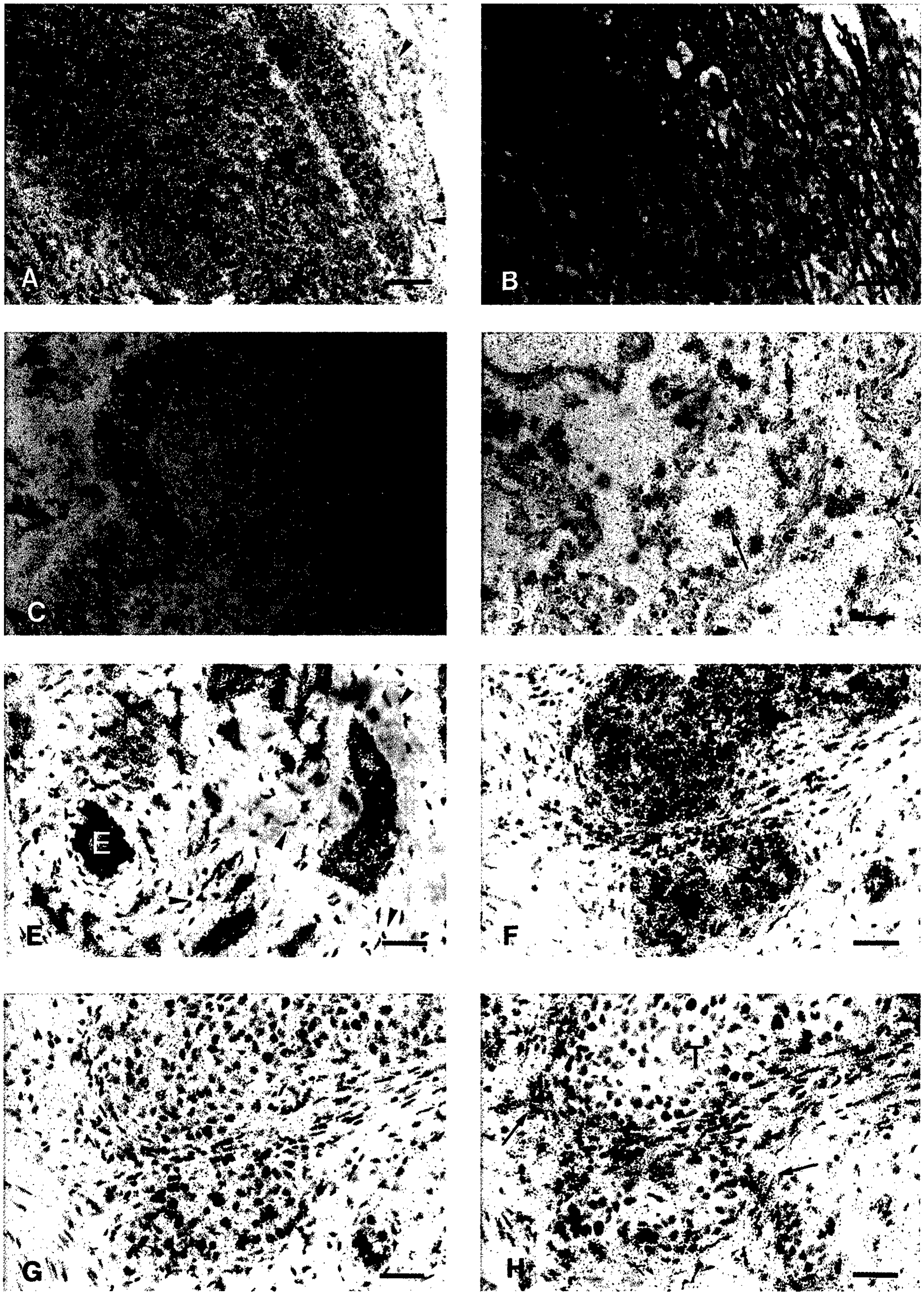

Figure 3 Localization of TCSF in lung cancers and breast cancers. **(A)** TCSF mRNAs are detected in invasive cancer cells (T) in lung carcinoma using an anti-sense probe, whereas stromal cells (arrowheads) are negative. Bar = 70 μm. **(B)** Same area treated with TCSF sense RNA probe. Bar = 70 μm. **(C)** Epithelial cells (E) of normal lung tissue do not express TCSF mRNA. (Bar = 70 μm). **(D)** TCSF mRNA is also present in many alveolar macrophages (arrow). Bar = 70 μm). **(E)** Intraductal breast tumor cells (T) express TCSF mRNAs, whereas epithelial adjacent cells (E) and stromal cells (arrowheads) do not show any hybridization grains. Bar = 70 μm. **(F)** Invasive tumor cells (T) express TCSF mRNAs, whereas stromal cells are negative. Bar = 70 μm. **(G)** Same area treated with TCSF sense RNA probe. Bar = 70 μm. **(H)** Gelatinase A mRNAs are localized in fibroblasts (arrow) in close contact to tumor clusters (T) in serial sections of breast carcinoma. Bar = 70 μm.

mor cell nests of both pre-invasive (1.68 ± 0.22 /μm^2) and invasive areas (2.14 ± 0.35 /μm^2) compared to the extracellular control compartment (0.19 ± 0.02 / μm^2) and normal tissue (0.23 ± 0.04 /μm^2) ($p<0.05$).

Breast Lesions

By Northern blotting, TCSF mRNAs were detected in the 22 breast carcinomas. Quantitative analysis showed significantly higher ($p<0.05$) expression of TCSF mRNAs in breast carcinomas than in benign breast lesions (Figures 1 and 2B). No significant differences between the TCSF mRNA levels were found in accordance with the Scarf and Bloom staging (Figure 2B).

With in situ hybridization, benign proliferations and normal mammary areas mixed with cancer cells or adjacent to cancer areas did not show any hybridization grains (Figure 3E), whereas the TCSF mRNAs were detected in cancer cells in pre-invasive and invasive areas (Figure 3F) of all 22 tumors examined. Stromal cells did not contain any hybridization grains. In the three cases studied by quantification, the density of markers in both pre-invasive (2.14 ± 0.48/μm^2) and invasive (2.95 ± 0.92/μm^2) areas was significantly higher than in the extracellular control compartment (0.23 ± 0.12/μm^2) and the normal areas (0.28 ± 0.10/μm^2) ($p<0.05$). On serial sections, TCSF mRNAs were localized in cancer cells, whereas fibroblasts close to tumor clusters expressed mRNAs encoding gelatinase A (Figures 3F and 3H) in the same areas.

Discussion

In this study we clearly showed the presence of mRNA encoding TCSF in epithelial tumor cells of lung and breast carcinomas. In agreement with our observations, previous immunohistochemical studies detected TCSF in cancer cells in breast (Zucker and Biswas 1994) and in bladder carcinoma (Muraoka et al. 1993). In the latter study, no staining was found in epithelial cells in non-neoplastic urothelium, except in superficial umbrella cells. However, Zucker and Biswas (1994) reported that TCSF is also present in normal breast ductules and lobules near in situ carcinoma areas, describing a more extensive distribution of TCSF compared with our data obtained by in situ hybridization (ISH). No TCSF transcripts were detected in normal epithelial cells in both non-neoplastic breast and lung lesions. It is therefore likely that normal epithelial cells express low levels of the TCSF mRNAs that we could not detect by ISH but that could produce amounts of the TCSF proteins detectable by immunohistochemistry. The presence of TCSF mRNAs in benign and normal tissues was confirmed by our Northern blot analysis, identifying weak expression of TCSF mRNAs in those samples. Furthermore, our Northern blot analysis strengthened our ISH data because a higher significant abundance of TCSF transcripts was found in both breast and lung carcinomas than in benign and normal samples. The detection of TCSF mRNAs in intraepithelial cancer areas of the lung and mammary gland indicates that TCSF mRNA overexpression is an early event in carcinogenesis. These data, taken together, suggest that the expression of TCSF mRNA can be correlated with tumor progression. However, despite its implication in cancer progression, TCSF might play a role in some other pathological processes, such as inflammation and emphysema, because it has also been detected in alveolar macrophages in our study and it has been proposed as a factor in arthritis (Karinrerk et al. 1992).

Even though the precise function of TCSF is not known, some recent in vitro studies have shown that TCSF is able to stimulate the production of several MMPs by fibroblasts. Recent experimental data have demonstrated that TCSF stimulates the production of interstitial collagenase, stromelysin 1 and gelatinase A but not stromelysin 3 in fibroblasts (Kataoka et al. 1993). However, stromelysin 3 is known to have a poor proteolytic activity against collagen-like substrates (Murphy et al. 1994). Moreover, these authors have also demonstrated that TCSF increases activation of gelatinase A. These results are of particular interest regarding the implication of TCSF in cancer progression, because in many carcinomas in vivo the stromal cells have been demonstrated to be the principal source of several MMPs. A variety of studies have indicated that fibroblasts adjacent to the malignant tumors clusters produce interstitial collagenase (Urbanski et al. 1992; Hewitt et al. 1991; Polette et al. 1991), stromelysins 1, 2, 3 (Polette et al. 1991; Basset et al. 1990), and MT-MMP1 (Polette et al. 1996; Okada et al. 1995). More precisely, in both lung and breast carcinomas, mRNAs encoding gelatinase A, which degrades basement membrane collagens (Tryggvason et al. 1993), have also been localized by ISH in the stromal cells surrounding invasive carcinomas (Polette et al. 1993,1994; Poulsom et al. 1992,1993; Soini et al. 1993). In addition to their specific localization at the tumor–stromal interface, MMPs were not or were only weakly found in normal tissues and benign lesions. These studies have therefore demonstrated an association between MMP expression and the invasive process in cancers. Using serial sections in breast carcinomas, we showed that there is an obvious expression of TCSF mRNAs by tumor cells and gelatinase A mRNAs by fibroblasts in the same areas.

It therefore appears that some MMPs, as well as TCSF, are expressed selectively in both pre-invasive and invasive carcinoma but by different cell types, peritumoral fibroblasts and tumor cells, respectively. Relating our in vivo data to the observation that TCSF

enhances the production of some particular MMPs in fibroblasts in vitro, it can be postulated that the TCSF produced by tumor cells in vivo stimulates the expression of some MMPs by peritumoral fibroblasts. However, in vivo, interstitial collagenase and stromelysin 1, which are induced by TCSF in fibroblasts in vitro, are infrequently observed in stromal cells in breast carcinomas (Polette et al. 1991), whereas they are present at high levels in lung cancers (Muller et al. 1991), despite the presence of TCSF in both types of carcinoma. Moreover, in four of 22 of our lung carcinomas, we failed to detect any TCSF transcripts. These findings are consistent with the involvement of other factors in the regulation of those MMPs, such as the origin of the tissue, the extracellular matrix environment, and genetic rearrangements.

In conclusion, our observations on lung and breast cancers strongly support the hypothesis that TCSF is an important factor in tumor progression. More precisely, TCSF produced by tumor cells could play a role in the degradation of extracellular matrix associated with tumor invasion by stimulating the synthesis of some MMPs by peritumoral fibroblasts.

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